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Prognostic Factors in Colorectal Cancer: Studies of Thymidylate Synthase Expression, Mismatch Repair Protein Expression, Tumor Budding and T-Cell Infiltration

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Prognostic Factors in Colorectal Cancer:
Studies of Thymidylate Synthase Expression, Mismatch Repair Protein Expression, Tumor Budding and T-Cell Infiltration

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To Yaya and my family.

ABSTRACT

Colorectal cancer (CRC) is a major health problem in the Western world. CRC is treatable with surgery and often curable when the disease is diagnosed at an early stage. With improved surgery and adjuvant chemotherapy treatment, the disease-free-survival (DFS) rate for colon cancer is approximately 80% for stage II and for stage III close to 65%. There is a continual need for the established risk stratification of CRC based on the tumor, node, metastasis (TNM) staging to evolve.

The overall aim of this thesis was to detect new potential prognostic and predictive factors for molecular and clinical responses in primary CRC. One specific aim was to establish whether thymidylate synthase (TS) expression in a large group of patients with stage II and stage III CRC is a prognostic and/or predictive factor alone or in combination with mismatch repair (MMR) status in the colon cancer subgroup. Another aim was to explore in primary CRC the potential association between tumor budding and MMR status and the impact of tumor budding as a factor of tumor recurrence and development of distant metastases. The last aim was to investigate the prognostic and predictive value of tumor budding, tumor border configuration and T-cell infiltration in primary colon cancer with known MMR status. Tumor material for the studies was derived from the adjuvant Nordic trials which randomized from 1991 to 1997, 2224 patients with stage II or stage III CRC to surgery alone or surgery plus adjuvant 5-FU-based chemotherapy. Immunohistochemistry (IHC) was used to detect and evaluate TS expression, MMR-status, tumor budding and infiltration of CD3+ and CD8+ T-cells.

Paper I. TS expression was assessed in tumors from 1,389 patients with stage II and III CRC. TS expression with the classification of TS grade 0-1 versus 2-3 as well as TS 1-2 versus TS 3 was prognostic in the surgery alone group. A high TS expression was associated with a shorter overall survival (OS). Among patients with TS grade 3, the subgroup treated with adjuvant chemotherapy had a significant longer OS compared with the group treated with surgery alone.

Paper II. Primary colon cancer with a deficient MMR status (dMMR) is associated with an improved prognosis. Data indicates that colon cancer with a proficient MMR status (pMMR) and with high TS expression has an improved response to adjuvant 5-FU-based chemotherapy. This study evaluated if a combined analysis of MMR status and TS expression in 716 colon cancer patients added prognostic value and better predicted response to adjuvant 5-FU-based chemotherapy. In the group of patients treated with surgery alone, patients with dMMR tumors and low TS grade had a trend to a better OS compared to patients with pMMR tumors and high TS grade. In stage III, patients with pMMR tumors and high TS grade who received adjuvant 5-FU-based chemotherapy had a significantly improved OS compared to patients treated with surgery alone. No relationship was found between MMR status and TS expression.

Paper III. A pMMR status compared to a dMMR status and tumor budding are considered adverse prognostic factors in primary CRC. This exploratory study included 134 patients with primary CRC. It assessed tumor budding grade in tumors with pMMR status compared to dMMR status to see if it differed as to whether local recurrence or metastases did or did not develop. The 29 available dMMR cases which developed recurrence or distant metastases (met+) were matched with a dMMR group with no recurrence or metastases (met-), and the pMMR/met+ group with pMMR/met- cases. A significantly higher percentage of high-grade tumor budding was only found in the dMMR/met+ group compared to the pMMR/met+ group.

Paper IV. Tumor budding is correlated to the development of local and distant metastases in CRC while high density of tumor infiltrating immune cells is associated with an improved prognosis. In this study, tumors from 478 patients with stage II-III colon cancer and known MMR status were examined to determine the prognostic value of tumor budding, tumor border configuration and CD3+ / CD8+ T-cell infiltration. An association was found between high grade tumor budding and more advanced stage, higher N-stage, pMMR status, an infiltrating tumor border configuration and lower levels of high score CD3+ and CD8+ T-cells. High grade tumor budding was correlated to worse OS in univariate analysis but not in multivariate. In the entire study population, an infiltrative tumor border configuration was an adverse prognostic factor for OS and a dense infiltration of CD8+ T-cells was independently associated with a better OS.

In conclusion, TS is a prognostic factor in CRC patients treated with surgery alone and patients with the highest level of TS expression had an improved clinical outcome following adjuvant 5-FU-based chemotherapy. In stage III colon cancer, a combined rather than a single marker analysis of MMR-status and TS expression, can improve the prediction of response to 5-FU-based chemotherapy. Whether tumor budding can provide prognostic information for patients with primary CRC and a dMMR status should be further explored in larger studies. An independent prognostic impact was found for CD8+ T cell infiltration and tumor border configuration as well as an association between them and tumor budding. Our study supports the inclusion of tumor border configuration, tumor budding, CD8+ T cell infiltration in the risk assessment for stage II-III colon cancer patients.

Key words: Colorectal cancer, colon cancer, TS, MMR, tumor budding, T-lymphocytes

Populärvetenskaplig sammanfattning

Cancer i tjock- och ändtarm (kolorektalcancer) är den tredje vanligaste tumörsjukdomen i Sverige och ett stort hälsoproblem i hela västvärlden. Oftast är kolorektalcancer botbar med kirurgi om den diagnostiseras i ett tidigt stadium. Med modern kirurgi och postoperativ cytostatikabehandling är sjukdomsfri överlevnad för koloncancer stadium II (tumören begränsad till tarmväggen) cirka 80 % och för stadium III (spridning till lymfkörtlar i närheten av tumören) cirka 65%. Risk för återfall beror till stor del på tumörens stadium vid diagnos. Ändå behövs kompletterande riskmarkörer för återfall. Det övergripande målet med avhandlingen var att studera nya sådana potentiella riskmarkörer som kan förutse dels risk för återfall i cancer och dels svar på behandling. Sådana markörer kallas prognostiska respektive prediktiva faktorer.

Tumörmaterialet för delarbete I-IV kommer från en nordisk studie som pågick under 1991–1997. Där randomiserades 2224 patienter med kolorektalcancer i stadium II-III till endast kirurgi alternativt kirurgi följt av postoperativ kemoterapi med 5-fluorouracil (5-FU).

I **delarbete I** studeras uttrycket av enzymet thymidylat syntetas (TS) i tumörmaterial från 1389 patienter med kolorektalcancer. TS är viktig för DNA-syntes och celltillväxt och blockeras av cytostatikabehandlingen med 5-fluorouracil (5-FU) vilket är en basbehandling vid kolorektalcancer.

Kroppen har flera sätt att reparera skador på DNA. Ett av dem är mismatch repair systemet (MMR). Defekter i MMR proteiner (dMMR) eller hög-mikrosatellitinstabilitet (MSI-H) leder till felaktig DNA-reparation. Detta uppstår i cirka 15% av kolorektalcancer. Patienter med kolorektalcancer som uppvisar dMMR har en bättre prognos jämfört med dem som har en MMR-stabil tumör (pMMR), men svarar sämre på adjuvant 5-FU terapi.
Studier har visat att patienter vars tumör uppvisar högt TS har kortare överlevnad men att de tenderar att svara bäst på 5-FU-baserad kemoterapi.

I **delarbete II** undersöks om en kombinerad analys av TS-uttryck och MMR-status har ett utökat prognostiskt- och prediktivt värde i ett material med 716 patienter med koloncancer i stadium II–III.

Tumor budding, som definieras som 1–5 isolerade tumörceller som lämnar tumörens invasionsfront, är associerad med utveckling av lokal- och fjärrmetastasering.

I **delarbete III** undersöks den potentiella associationen mellan tumor budding och MMR-status som en faktor för lokalrecidiv respektive fjärrmetastasering i en grupp om 134 patienter.

Utseendet av tumörens invasionsfront har betydelse för prognos. Förekomst av immunceller, till exempel CD3+ och CD8+ T-celler, i tumörvävnad är associerat till en bättre prognos.

I **delarbete IV** utforskas det prognostiska värdet av tumor budding, utseende av tumörens invasionsfront och T-cell infiltration (CD3+ och CD8+) i en grupp bestående av 478 patienter med koloncancer.

För att påvisa TS uttryck, MMR och förekomsten av tumor budding samt T-lymfocyt infiltration av CD3+ och CD8+ T-celler användes immunohistokemi.

Resultat i delararbete I-IV

Delarbete I: Studien visade att TS-uttryck var prognostiskt i den grupp av patienter som endast fick kirurgi som behandling. Högt TS-uttryck var associerat med en sämre prognos. I gruppen med högt uttryck av TS, hade patienter som fått kemoterapi en signifikant längre överlevnad jämfört med patienter med endast kirurgisk behandling.

Delarbete II: Ingen korrelation noterades mellan TS-uttryck och MMR-status. I gruppen med pMMR och högt TS-uttryck fanns hos stadium III patienter en signifikant förlängd överlevnad i gruppen som fått kemoterapi jämfört med gruppen som endast behandlades kirurgiskt.

Delarbete III: Studien visade att gruppen med dMMR/MSI-H som utvecklade lokalt recidiv alternativt fjärrmetastaser hade en hög frekvens tumor budding i sina tumörer.

Delarbete IV: En koppling hittades mellan hög buddingfrekvens och en infiltrerande invasionsfront, ett mer avancerat stadium, pMMR-status och lägre infiltration av CD3+ och CD8+ T-celler. Invasionsfrontens utseende och CD8+ T-cell infiltration var oberoende prognostiska faktorer. En infiltrerande invasionsfront var associerat med sämre prognos medan hög infiltration av CD8+ T-celler var associerat med förbättrad prognos.

Sammanfattning
TS är en prognostisk faktor för patienter med kolorektalcancer som behandlas endast med kirurgi. Med en kombinerad analys av både MMR och TS bör man bättre kunna förutse svar på cytostatikabehandling efter kirurgi. Större studier bör genomföras för att se om tumor budding kan ge kompletterande prognostisk information till patienter vars koloncancer uppvisar dMMR. Vår studie stödjer inkludering av analys av tumörens invasionsfront, tumor budding och framförallt CD8+ T-cell infiltration i riskvärdering och riskanalys för kolorektalpatienter i stadium II–III.

LIST OF SCIENTIFIC PAPERS

I. Karlberg M, Öhrling K, Edler D, Hallström M, Ullén H, and Ragnhammar P. Prognostic and predictive value of thymidylate synthase expression in primary colorectal cancer. *Anticancer Res 30: 645-652, 2010.*

II. Öhrling K, Karlberg M, Edler D, Hallström M, and Ragnhammar P. A combined analysis of mismatch repair status and thymidylate synthase expression in stage II and III colon cancer. *Clin Colorectal Cancer 12: 128-135, 2013.*

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IV. Karlberg M, Mezheyeuski A, Stenstedt K, Hallström M, Lenander C, Ling A, Palmqvist R, Edler D. Prognostic value of tumor budding, tumor border configuration and T-cell infiltration in colon cancer. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
ASCO	American Society of Clinical Oncology
CH2THF	5-10-methylene tetrahydrofolate
CEA	Carcino-embryogenic antigen
CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CRM	Circumferential resection margin
CSS	Cancer specific survival
DAB	Diaminobenzidine tetrahydrochloride
DFS	Disease-free survival
DPD	Dihydropyrimidine dehydrogenase
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EPOC	European Organization for Research and Treatment of Cancer
ESMO	European Society for Medical Oncology
5-FU	5-Fluorouracil
FAP	Familial adenomatous polyposis coli
H&E	Hematoxylin and eosin
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Hazard ratio
IARC	International Agency for Research on Cancer
IFN	Interferon gamma
IGF	Insulin growth factor
IHC	Immunohistochemistry
IS	Immunoscore
LAT	Local ablative treatment
LOH	Loss of heterozygosity
LV	Leucovorin
mCRC	Metastatic Colorectal Cancer
MDT	Multidisciplinary team
MGMT	Methyl guanine methyltransferase
MHC	Major Histocompatibility Complex
MMR	Mismatch repair
MRF	Mesorectal fascia
MRI	Magnetic Resonance Imaging
MSI	Microsatellite instability
MSI-H	Microsatellite instability high
MSI-L	Microsatellite instability low

MSS	Microsatellite stable
NK	Natural Killer
OMD	Oligometastatic
OS	Overall Survival
PCR	Polymerase chain reaction
PFS	Progression-free survival
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
RT	Radiotherapy
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEER	Surveillance Epidemiology and End Results
SNP	Single Nucleotide Polymorphism
TGF	Transforming growth factor
TH	T Helper cell
TILs	Tumor infiltrating lymphocytes
TMA	Tissue microarray
TME	Total mesorectal excision
TNM	Tumor, node, metastasis
TP	Thymidine phosphorylase
Treg	Regulatory T-cell
TS	Thymidylate synthase
UICC	International Union Against Cancer
VEGF	Vascular endothelial growth factor

1. BACKGROUND

1.1 Epidemiology and Etiology

Cancer of the colon or rectum is a major health problem. Worldwide it is considered the third most common cancer accounting for approximately 880 792 deaths per year, an estimated 1.8 million new cases and 1.2 million deaths in the coming ten years [1, 2].
Approximately two thirds of the cases are colon cancers and the rest are rectal cancers [3]. Colorectal cancer (CRC) ranks second worldwide with regard to mortality [2]. In Sweden the incidence rate of colorectal cancer (CRC) in 2017 was 69/100 000 [4] (Table 1). There is a higher incidence of CRC, about two thirds, in developed countries compared to developing countries which is attributed to a western lifestyle [3].

Table 1. Colorectal cancer incidence (total number) in Sweden 2017

Tumor site	Male	Female	Total
Colon	2324	2313	4637
Rectum	1383	992	2375
Total	3707	3305	7012

CRC occurs relatively seldom (<5%) in persons younger than 40 and increases thereafter [5]. A stabilizing or decreasing trend in incidence and mortality of CRC has been identified in highly developed countries which is most likely due to early detection of the tumor, improved surgical techniques as well as oncological treatment [1, 3].

Screening programs for early detection of CRC based on fecal occult blood testing and colonoscopy are currently underway in most European countries, Canada, North America [6]. Analyses of the first five years of the CRC screening program established in Stockholm, Sweden 2008 for men and women age 60-69 showed that 1.8% were examined with colonoscopy and 0.1% were diagnosed with CRC [7]. There is some more recent data indicating a CRC incidence increase in left-sided tumors, especially rectal cancer, in younger adults, under the age of 50 [3, 8]. These younger patients tend to present with a more advanced stage at diagnosis and a worse outcome [9]. The cause of the increase in young adult CRC is unknown but it is estimated that 35% are associated with known hereditary CRC syndromes [8, 10, 11]. An estimate was done in a retrospective cohort study of the Surveillance, Epidemiology, and End Results Reporting (SEER) CRC registry predicting an increase of incidence rates by 2030 in colon and rectal cancer for patients age 20 to 34 years to 90.0% and 124.2%, respectively [12].

In general, an interesting shift has been observed internationally with a greater increase in right-sided colon tumors, especially cecal ones [13, 14]. This is thought to be due in part to colonoscopy being a better diagnostic tool for left-sided tumors due to anatomical reasons, differing tumor biology of the right-sided tumors (flatter tumors such as serrated adenomas) as well a true increase in incidence of right-sided tumors [15].

The development of CRC of which more than 90% are adenocarcinomas is a multifactorial process with its cause encompassing genetic predispositions, environmental exposures and inflammatory conditions of the digestive tract [16].

1.2 Risk Factors

Influencing screening recommendations
Increasing age is a known risk factor for sporadic CRC as mentioned above. Individuals with a long-standing inflammatory bowel disease, ulcerative colitis or Crohn's disease have an increased risk of developing colorectal cancer and are included in special programs for screening and follow-up [17].

1.2.1 Genetic risk and family history

CRC is considered in most cases (75-80%) a sporadic disease. Approximately 2-8% of CRC is caused by genetic predisposition, due to pathogenic germline variants in genes associated with high cancer risk [18, 19]. Lifetime risks for CRC can approach 50-80% for mutation carriers in the absence of endoscopic and/or surgical intervention. [20].

1.2.2 Polyposis syndromes

Familial adenomatous polyposis (FAP) is a rare inherited autosomal dominant condition that is characterized by the presence of hundreds to thousands of polyps in the colon and rectum [21]. It is characterized by a germline mutation in the adenomatous polyposis coli (APC) gene, a tumor suppressor gene (linked or located on chromosome 5q21). It is an early mutation in the development of colorectal cancer and is the initiating event in the chromosomal instability (CIN) pathway to colorectal tumorigenesis. The CIN (APC) pathway is also a common molecular pathway for sporadic CRC. Approximately 1% of all CRC is caused by FAP and if untreated it inevitably leads to cancer [21]. Tumors are mostly located in the distal colon and rectum. FAP patients are screened regularly for CRC beginning at around the age of 10 to 12 with colonoscopies and colectomy is performed electively in the late teens/early twenties or earlier depending on symptoms or with suspected/verified CRC [22].

Another polyposis associated syndrome is the MUTYH-associated polyposis (MAP) which is as autosomal recessive disease due to germline mutation to the base excision repair gene MUTYH1. There is a carrier frequency of 1% in the European ancestry population and it is associated with a moderate 1.5-2 fold increased risk for CRC, especially if there is a first degree relative with CRC [23].

Syndromes which also have a greater risk for CRC are Peutz-Jeghers syndrome (PJS), Juvenile polyposis syndrome (JPS) and the phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-hamartoma tumor syndrome (PHTS) [24].

1.2.3 Non-polyposis syndrome

Lynch Syndrome, previously referred to as Hereditary Nonpolyposis Colorectal Cancer (HNPCC), is an autosomal dominant cancer predisposition syndrome caused by a germline mutation in one of the four mismatch repair (MMR) genes (MLH1, PMS2, MSH2, MSH6) or within the epithelial cell adhesion molecule (EPCAM) gene adjacent to the MMR gene, MSH2 [25, 26].

In contrast, deficiency in MMR for sporadic CRC which is present in 15-20% of all colon cancer, is mainly due to an epigenetic inactivation by the biallelic methylation of the MLH1 gene promoter [27]. Defective MMR (dMMR) leads to failure to repair errors during replication of small repetitive DNA sequences, microsatellites, and thus induces microsatellite instability (MSI) [28].

MLH1 and MSH2 gene mutation carriers have a higher lifetime risk, between 30-74%, of CRC compared to gene mutation carriers of MSH6 and PMS2 that have a risk range of 10-20% [25]. Male mutation carriers have a higher lifetime risk for CRC than females. In Lynch syndrome 60-80% of the tumors are located in the proximal colon (proximal to the splenic flexure) [29].

About 3% of newly diagnosed cases of CRC are caused by Lynch syndrome. It is considered a multi-tumor syndrome in which the most common malignancy is CRC and the second most common is endometrial cancer while other tumor sites such as stomach, biliary tract, ovaries and brain are less common [30]. There is a 70-90% lifetime risk for cancer in mutation carriers with development of cancer at a mean age of about 45 years and a risk of multiple synchronous and metachronous tumors [31]. The Amsterdam criteria as well as the Bethesda guidelines have been developed to identify and classify families with the Lynch syndrome [32]. High risk individuals should undergo colonoscopy with (1)-2 year intervals from the age 20-25 years and upon a CRC diagnosis a subtotal colectomy may be recommended due to the high-risk for metachronous tumors [30, 31].

Although germline mutation analysis on blood samples is the gold standard in Lynch syndrome diagnostics it is not feasible as a universal screening method due to cost and time-consumption. In tumors with immunohistochemical loss of MLH1/PMS2 further BRAF mutation analysis may aid in distinguishing somatic alterations from germline defects. Lynch syndrome CRCs can present with KRAS mutations but not BRAF mutations while BRAF mutations (mostly in V6000 codon) occur exclusively in sporadic dMMR tumors or MSI-high (MSI-H) tumors [33].

Proficient MMR (pMMR) hereditary nonpolyposis CRC has been detected in half of the CRC families meeting the Amsterdam criteria. No identifiable germline mutations have been found in the MMR genes. The lifetime risk for CRC is lower for this group and no increase in extracolonic tumors has been seen [34].

1.2.4 Familial syndromes

Excluding the FAP and Lynch syndrome, carcinomas and adenomas aggregate in families in which the specific genes have not been found. There is an approximate two-fold risk of developing CRC for an individual with a first-degree relative with CRC and four-fold risk with at least two affected relatives [35, 36]. About 10 to 30% of CRC cases are thought to be attributed to this syndrome.

1.2.5 Other potential risk factors (*modifiable*)

Extensive research has been done in order to determine the importance of environmental factors in the development of CRC.

Obesity has been found to be a risk factor for CRC [37]. A link was found between diabetes and the risk of developing CRC in a meta-analysis of 14 studies [38]. A theory of the mechanism behind is that high levels of insulin-like-growth factor (IGF-1) and IGF binding protein-3 (IGFBP-3) stimulate colonic tumor cells [39].

The use of tobacco has been associated, especially in rectal cancer, with increased incidence and mortality from CRC [40].

An excessive alcohol consumption is also considered a risk factor for CRC [41]. This could be due to a decreased folate intake caused by alcohol interfering with folate absorption but the role of folate as a protective agent against CRC is controversial [42].

Long-term consumption of red meat or processed meat has been implicated with a higher risk for CRC, in particular left-sided tumors [43].

There is data suggesting an association between diet and the development of CRC mediated by a decreased diversity of the gut bacterial microbiome and increased colonization of the gut by strains of Fusobacterium or E. coli [44].

1.2.6 Factors that can reduce risk

The intake of a diet high in fiber, fruits and vegetables has been shown in some epidemiologic studies to decrease the risk for developing CRC [45].
An active lifestyle was shown in a study to decrease the risk of colon cancer but not for rectal cancer [46].

Long-term use of aspirin or nonsteroidal anti-inflammatory drugs (NSAID) protect against CRC, particularly proximal colon tumors [47]. Studies have shown that aspirin impairs tumor cell growth by inhibition of cyclooxygenase-2 (COX-2) and increased apoptosis [48]. This protective effect was also seen in a study in Lynch syndrome patients [49].

COX-2 is an enzyme encoding for prostaglandins which in turn control inflammation processes and are known to promote cellular proliferation, migration, invasiveness, angiogenesis. COX-2 overexpression is associated with decreased survival in CRC patients which ties into the concept of cancer-related inflammation being a hallmark of cancer. Epidemiological studies noted that benefit of aspirin after CRC diagnosis was limited to patients with PIK3CA-mutated tumors [50]. About 15-20% of CRC patients harbor mutations in PI3KCA [51]. Upon mutation of the PIK3CA gene which encodes PI3K kinase, a constitutive activation occurs of the PI3K-AKT-mTOR pathway which plays a role in stimulating carcinogenesis. This also results in COX-2 upregulation [50].

A randomized, double-blinded, placebo controlled study (ALASCCA) on stage I-III CRC, is currently ongoing in Sweden and studies the post-operative protective effect of daily low dose aspirin versus placebo in patients with mutated PI3KCA tumors [52].

2 MOLECULAR PATHOGENESIS OF COLORECTAL CANCER

2.1 Adenoma to Carcinoma Progression Sequence

The majority of CRCs are believed to develop from adenomas (adenomatous polyps) that evolve to carcinomas over 10 to 15 years. Removal of polyps is confirmed in controlled trials to decrease the incidence of CRC [53]. The adenoma-carcinoma sequence was described by Fearon and Vogelstein in 1990 as a genetic multistep model for colorectal carcinogenesis which encompasses the accumulation of mutations in the adenomas, with mutation in the tumor suppressor gene, APC, occurring early, followed by mutation of the oncogene KRAS and inactivation of the tumor suppressor gene p53 occurring late [54].

In the original model it was proposed that only tubular and tubulovillous adenomas had the potential to progress to invasive adenocarcinoma. It is now known that even serrated polyps can become malignant via an alternative pathway, the CIMP pathway, which differs from the APC tumor-suppressor gene pathway associated with the conventional tubular adenomas [55]. Colorectal cancer is considered a heterogeneous disease in which the different pathways to malignancy can be distinguished at the molecular level by the genomic and epigenomic instability. In addition to point mutations, other genetic changes occur such as altered DNA methylation (epigenetic), gene rearrangements, amplifications, overexpression and deletions. It includes the activation of oncogenes, genes that partake in cell growth pathways and cell cycle regulation and deactivation of tumor suppressor genes, genes that suppress the cell cycle [56].

The three most common pathways leading to CRC are: the chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) pathways [57]. Figure 1 illustrates these pathways, including the molecular pathogenesis, leading to the development of CRC [54].

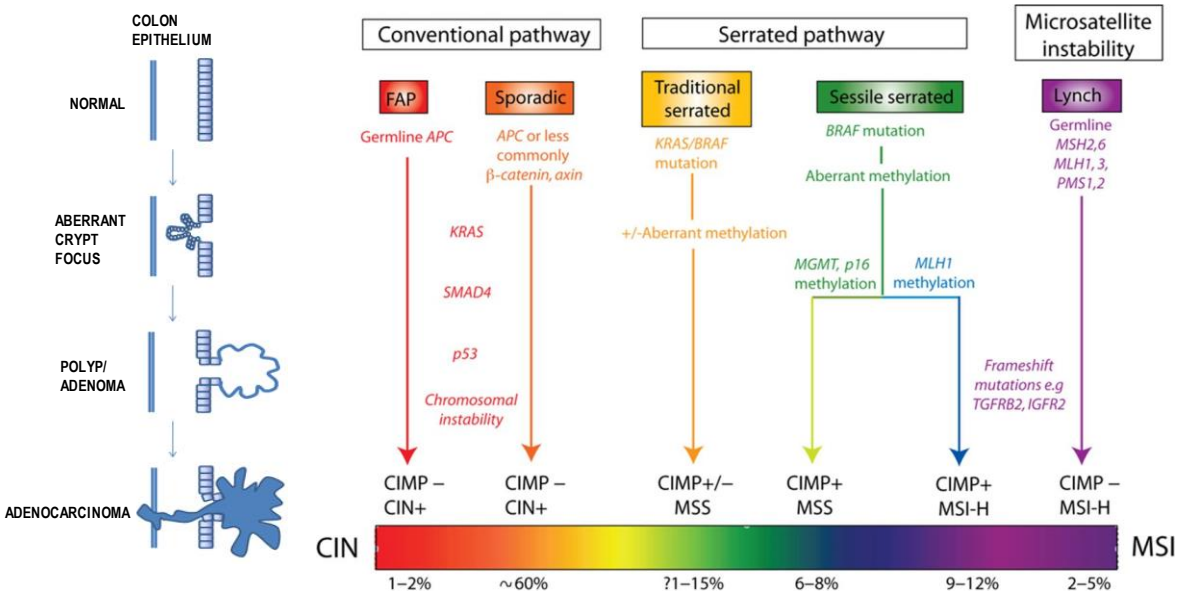


Figure 1. Pathways leading to CRC. *(Reprinted, with permission, from East et al. Gut 2017)*

2.2 Chromosomal Instability (CIN) Pathway

The CIN pathway accounts for the development of as many as 85% of the sporadic CRCs [58]. CIN is believed to increase clonal diversity in tumor cells thus stimulating cancer progression.

Typical for the CRC CIN phenotype is a distal tumor location, a well-differentiated (low-grade) tumor, a higher tendency to local lymph node and distant metastases as well as a low or no peritumoral lymphocyte infiltrate.

2.2.1 APC

The earliest event in the CIN pathway is the genetic disruption of the APC gene, located on chromosome 5q. As mentioned previously, germline mutations in APC are responsible for FAP while somatic mutations in APC are observed in the sporadic CRCs. Other than APC, there are several deregulated genes in CIN that affect chromosome segregation, telomere regulation and DNA damage response.

The APC gene product is a protein involved in functions such as regulation of differentiation, adhesion, polarity, migration, development, apoptosis and chromosomal segregation [59]. In its normal state the APC protein interacting with glycogen synthase kinase-3β (GSK-β), casein kinase 1α/ε (CK1α/ε) and β-catenin provide suppression of the Wnt signal. A genetic disruption of APC is the first event which in turn constitutively activates the Wingless/Wnt signaling pathway. The mutant APC gene product cannot bind to β-catenin, a protein encoded by the CTNNB1 gene, which leads to increased cytoplasmic levels of β-catenin allowing it to translocate to the nucleus and activate transcription of multiple genes involved in tumor growth and invasion, see Figure 2 [59].

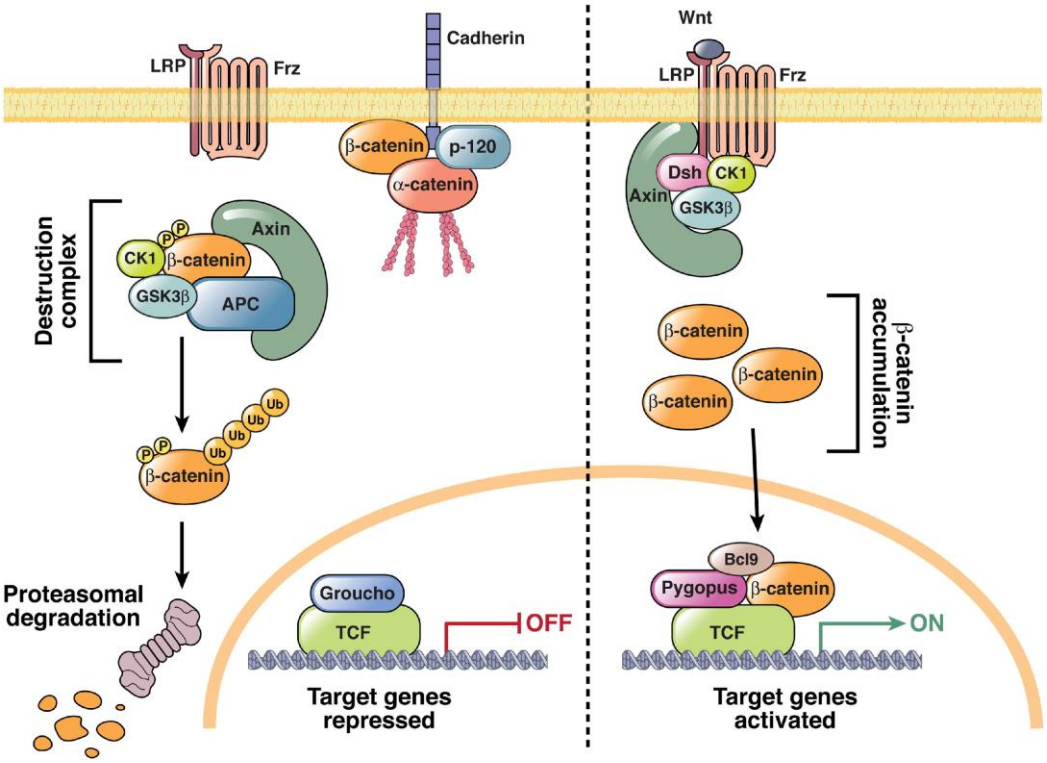


Figure 2. The Wnt Signaling pathway in the "OFF" and "ON" states. *(Reprinted, with permission, from Pino et al. Gastroenterology 2010)*

"OFF": in the absence of a Wnt signal, the destruction complex containing adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β) and casein kinase 1α/ε (CK1α/ε) on an axin-conducting scaffold targets the degradation of cytoplasmic β-catenin in a proteasome-dependent manner. In the nucleus, Wnt target genes are also kept silent by the repressor Groucho interacting with DNA-bound T cell factor (TCF).
"ON": in the presence of a Wnt ligand, occupancy of the receptors Frizzled (Frz) and coreceptor low-density lipoprotein receptor-related protein (LRP) triggers the phosphorylation of the cytoplasmic tail of LRP by CK1 and GSK-3β as well as the disheveled (Dsh)-dependent recruitment of axin on phosphorylated LRP. Phosphorylation of β-catenin no longer occurs, and the increased cytoplasmic levels of β-catenin translocate to the nucleus, where the transcription of multiple genes is initiated through displacement of Groucho and the interaction of β-catenin with the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors.

2.2.2 P53

The p53 gene, a key tumor suppressor gene, is located on chromosome 17p and is considered the 'guardian of the genome' [60]. It is a stress-inducible transcription factor regulating many different downstream genes that have a regulative function in several signaling processes. Stress stimuli such as DNA-damage response, dysfunctional telomeres, oncogene activation and oxidative stress, trigger p53 to induce cell cycle arrest, apoptosis or senescence [61]. p53 is the most commonly mutated gene in human cancers [62]. In sporadic CRC p53 mutation occurs in approximately 40-50% with a higher frequency in distal tumors compared to proximal tumors [63]. The loss of p53 function is a late event and is believed to play a critical role in the adenoma-carcinoma transition.

CRC patients with a mutant p53 seem to be more chemo-resistant and have poorer prognosis compared to patients with non-mutated/wild-type p53 CRC [64]. The p53 tumor suppressor function is in part due to its inhibition of the Wnt pathway and epithelial mesenchymal transition (EMT) through miR-34. Thus, loss of this inhibition by disruption of p53 could activate proliferation and tissue invasion by CRC cells [65].

2.2.3 Other CIN events

Typical for CIN pathway CRCs is a widespread imbalance in chromosome number (aneuploidy), loss of heterozygosity (LOH) with the majority (as many as 70%) of losses on chromosome 18 which contain the tumor suppressor genes DCC, SMAD4 and SMAD2 [66]. The loss of DCC expression has been associated with worse prognosis in stage II CRC [66].

Somatic mutations in SMAD4 have been found in some CRC patients [67]. Patients with juvenile polyposis syndrome have germline mutations in SMAD4 and have an increased risk of CRC [68]. The SMAD4 gene protein product is necessary for the function of the signaling pathway of the transforming growth factor-beta (TGF-β) which has a suppressive effect on cancer cells. Interference with TGF-β signaling allows cancer cells to escape this inhibition [69].

There is evidence that the erosion of telomeres plays a role in CIN. Telomeres are DNA-protein complexes of hexameric repeats (TTAGGG) that protect the ends of eukaryotic chromosomes during chromosome segregation. A theory is that telomere shortening stimulates CIN in early carcinogenesis while in later stages activation of telomerase, the enzyme complex that elongates telomeres, contributes to the immortality of tumor cells [70].
Research focuses on identifying mutations in key genes (e.g. APC, CTNNB1, BRAF, KRAS) and their signaling pathways contributing to progression of cancer. The signaling

pathway involved in tumorigenesis such as the Ras-Raf-MEK-ERK signaling pathway will be discussed later on in section 7.1 and in the CRC oncological treatment section (6.2.4) with regard to KRAS and BRAF and their clinical implications. The PI3K/Akt/mTOR pathway (section 6.7.1) and the Wnt/β-catenin signalling (section 2.2.1 and 7.4.1) will also be discussed later on.

2.3 Microsatellite Instability (MSI) Pathway

DNA repair mechanisms are essential for a cell's genetic stability and function. The damage on one of the DNA strands can be correctly repaired by excision and then replaced by synthesizing new DNA using the complementary strand as a template. Three main excision methods used are base excision repair (BER), nucleotide excision repair and mismatch repair (MMR) [71]. MMR genes include: MLH1 (mutL homolog 1), PMS1 (postmeiotic segregation 1), PMS2 (postmeiotic segregation 1), MSH6 (mutS homolog 6) and MSH2 (mutS homolog2).

In humans, the protein products of these genes function as two heterodimers, MutS and MutL. Specifically the MutSα complex is formed by MSH2/MSH6 and the MutSβ by MSH2/MSH3 while the MutLα complex consists of MLH1/PMS2 and the MutLγ of MLH1/MLH3. MutSα has the ability to recognize single base-pair mismatches as well as single-base insertion/deletion loops (IDLs) and the MutSβ recognizes larger IDLs (2 to 8 nucleotides). A known interaction that enables the DNA damage correction process is the binding of MutSα to MutLα. The function of the interaction of MutSβ to MutLγ is unclear. Once the MutSα complex recognizes the single base pair mismatch it slides as a clamp around the DNA strand and recruits MutLα. Upon reaching the DNA polymerase complex, it becomes a large complex by further interaction with exonuclease I and proliferating cell nuclear antigen (PCNA) and can thus excise nucleotides in the daughter strand back to the mismatch. Thereafter, the sliding complex disassociates leaving the DNA strand available for DNA resynthesis [72]. Figure 3 illustrates the steps involved in MMR [72].

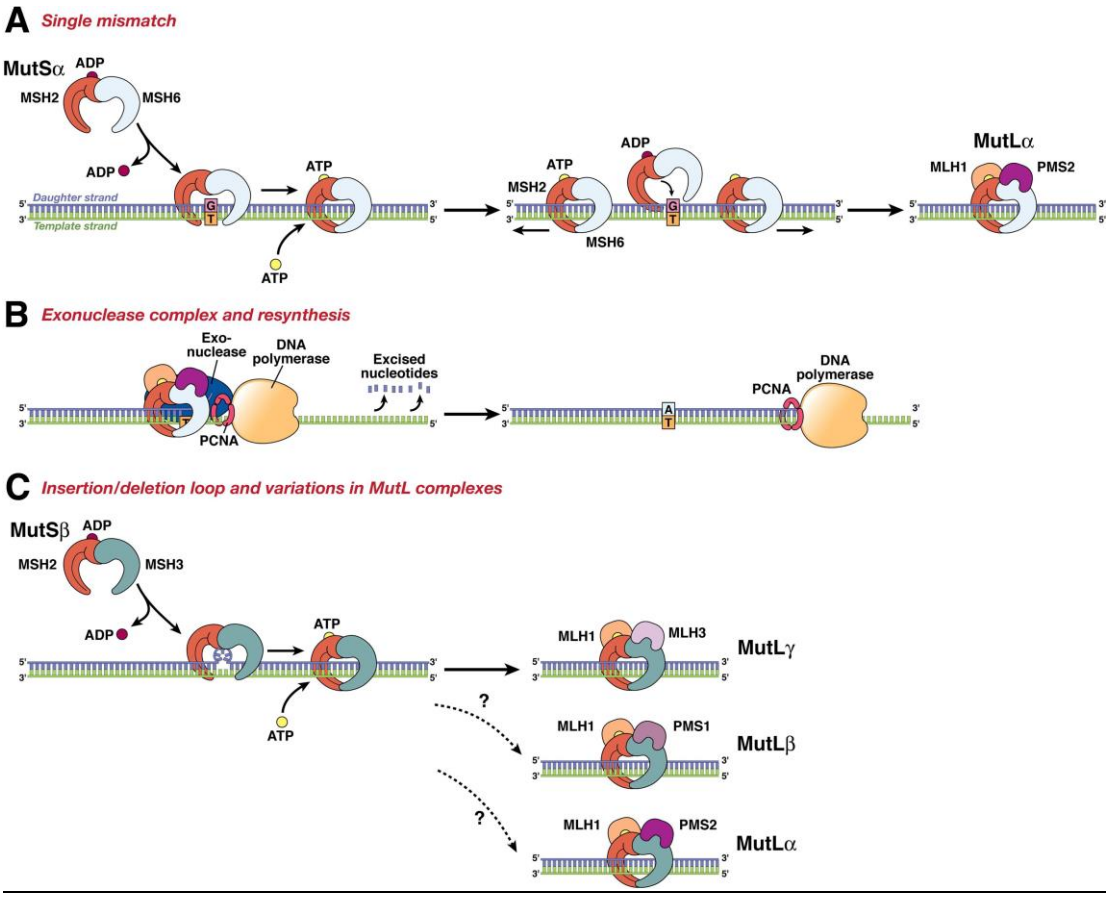


Figure 3. DNA MMR system. *(Reprinted, with permission, from Boland et al. Gastroenterology 2010)*

Step (A): MSH2-MSH6 (MutSα) recognizes single base-pair mismatches (i.e. DNA polymerase has matched the wrong base G with the T on the template) and creates a sliding clamp around the DNA. This requires the exchange of adenosine triphosphate (ATP) for adenosine diphosphate (ADP) (by MSH2, but neither MSH6 nor MSH3). The complex diffuses away from the mismatch site and is then bound by the MLH1-PMS2 (MutLα) complex. This larger (MutSα/MutLα)-complex (sliding clamp) moves along the new DNA chain until it encounters the DNA polymerase complex.

Step (B): The DNA MMR protein sliding clamp interacts with exonuclease-1, proliferation nuclear antigen (PCNA), and DNA polymerase. Excision then occurs of the daughter strand back to the site of the mismatch and eventually the complex falls off the DNA and resynthesis occurs, correcting the error.

Step (C): Variations on the DNA MMR interaction: MSH2-MSH6 recognizes single pair mismatches and small insertion-deletion loops (IDLs). MSH2-MSH3 (MutSβ) recognizes larger IDLs. MLH1 can dimerize with PMS2, PMS1, or MLH3. MSH2-MSH3 (MutSβ) prefers to interact with MLH1-MLH3 (MutLγ) but other heterodimers exist and are not entirely understood.

Microsatellites are defined as short tandem repeats of DNA sequences (binucleotide repeats) located throughout the genome and are distinctive and consistent in length in every tissue in each person [72]. The inactivation of the MMR genes results in a deficient MMR (dMMR) which then causes failure in the correction of insertion or deletion of the repeating units (microsatellites) during DNA replication. MSI leads to frame-shift mutations and premature stop codons and inactivation of genes involved not only in DNA repair (e.g. MMR genes) but even in cell proliferation (e.g. IGFR2, TGF-β) and in cell cycle/apoptosis (e.g. BAX, PTEN). This mutational signature is used to identify tumors with MSI [72].

MSI in colorectal cancer is observed in approximately 15% of cases where 3% of the cases are due to Lynch syndrome caused by germline mutations in one of MMR genes as

mentioned previously and 12% are sporadic cases [72]. Contrary to CIN tumors, also referred to as microsatellite stable (MSS) tumors, MSI tumors exhibit a diploid karyotype and the gene mutations differ from those observed in CIN CRCs. There seems to be a subgroup of CRCs that display both CIN and MSI [73].

The majority of tumors that are MSI-H have deficient MMR while most MSI-low (MSI-L) tumors have no MMR defect. Downregulation of MSH3 occurring heterogeneously throughout the tumor is believed to induce MSI-L [72].

Most sporadic MSI-H CRC is not due to a mutation in a MMR gene but to the loss of MMR activity due to the epigenetic event of hypermethylation of a gene promoter for a MMR gene (often the MLH1 gene) in patients with the CpG island methylator phenotype [74]. Epigenetics involves modifications in the phenotype or gene expression and does not indicate changes of DNA sequence but rather transcriptional silencing of gene expression.

Sporadic MSI CRC has been found to be associated with the serrated neoplasia pathway and often have $BRAF^{v600E}$ mutations differing from Lynch tumors which do not have BRAF mutations and present only with KRAS mutations [75].

Contrary to CIN tumors, MSI CRC are frequently located in the right colon, tend to be mucinous with signet ring cell morphology, poorly differentiated (high-grade), have strong lymphocyte infiltration and a pushing border configuration.

2.3.1 Methods to detect MSI-high (MSI-H)

A technique used widely to measure MSI is polymerase chain reaction (PCR) which creates thousands to millions of copies of a particular segment of DNA. The amplification of amount of the particular DNA sequence aids in its detection [76]. Of the several microsatellite loci that could be used for MSI analysis a National Cancer Institute (NCI) workshop recommended in 1997 a reference panel (Bethesda panel) consisting of 5 markers (2 mononucleotide: BAT-225 and BAT-26 and 3 dinucleotide: D2S123, D5S346, D17S250) [77]. In their guidelines for MSI classification MSI-H is defined as instability in 2 or more of these markers while microsatellite stable (MSS) and MSI-L as instability in only one of the markers. Currently many laboratories use a panel of 5 mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) that have high sensitivity and specificity for recognizing defective DNA in the MMR genes and don't require corresponding normal tissue [78].

In the clinical setting, immunohistochemistry (IHC) which is equivalent to MSI analysis in specificity and sensitivity, is often more available to identify the absence of expression of MMR proteins and thus identify the specific MMR gene that should be sequenced [79]. More detailed information on IHC analysis is given in section 9.2.

2.4 CpG Island Methylator Phenotype (CIMP) / Serrated Pathway

DNA methylation has an essential role in the alteration of gene expression seen in carcinogenesis and is extensively researched as biomarkers of CRC [80].

Epigenetic instability in CRC is exhibited as hypermethylation of loci containing CpG islands and as global hypomethylation. A subgroup of CRCs, 10-20%, have a very high proportion of methylated CpG loci and are considered CIMP+ tumors [81]. It is unclear which mechanisms cause CIMP. The aging process which causes an epigenetic drift could contribute to an increased aberrant DNA methylation inactivating a tumor suppressor gene resulting in the development of CRC [82].

The CIMP+ defect in colorectal tumors often results in hypermethylation of CpG islands in the promoter region of the MMR gene, MLH1, silencing its gene expression thus resulting in the dMMR/MSI-H phenotype seen in sporadic CRC [83]. Epigenetic alteration can inactivate several cellular pathways including not only DNA repair system (MLH1 and also MGMT) but also apoptosis (DAPK), angiogenesis inhibition (THBS1), metastasis suppression (TIMP3), cell cycle regulation (p14 ARF, p15 INK 4b, p16 INK4a) and cell adherence (CDH1, CDH13) [84].

The criteria for determining CIMP is increased methylation in at least three foci from a selected panel of five gene-associated CpG islands. Subclasses of CIMP are suggested in which CIMP low has <2/5 markers and CIMP high has >3/5 markers [85].

There is a correlation between CIMP-high CRC, deriving most likely from sessile serrated polyps, and right colon cancer, MSI-H and a high rate of $BRAF^{V600E}$ mutations but no KRAS mutation. CIMP-low is associated with MSS and carries mutant KRAS [86].

2.5 Summary Genetic and Epigenetic Events in CRC Development

In summary there are several genetic and epigenetic events involved in the development of CRC. A summary of the genes involved and molecular alterations is in Table 2 [59].

Table 2. Genetic and epigenetic events in the development of CRC

Tumor Suppressor Genes	**Proto-Oncogenes**	**Other Molecular Alterations**
APC(a)	BRAF	Chromosome instability
ARID1A	ERBB2	CpG island methylator phenotype
CTNNB1 (b)	GNAS	Microsatellite instability
DCC (c)	IGF2	Mismatch-repair genes
FAM123B	KRAS (f)	SEPT9
FBXW7	MYC	VIM, NDRG4, BMP3
PTEN	NRAS	POLE/POLD1
RET	PIK3CA (g)	
SMAD4(d)	RSPO2/RSPO3	
TGFBR2	SOX9	
TP53(e)	TCF7L2	

Genetic mutations in CIN-positive CRC:
(a) *5q21; **30-70%, ***inhibition of Wingless/Wnt signaling, cytoskeletal regulation
(b) *3p22; **~4-15% (~50^), ***regulation of Wnt pathway target genes that promote tumor growth and invasion
(c) *18q21; **~6%; ***cell surface receptor for netrin-1
(d) *18q21; **~10-20%; ***intracellular mediators of the TGF-β pathway
(e) *17p13; **~40-50%; ***cell cycle arrest, apoptosis induction
(f) *12p12; **~30-50%; ***cell proliferation, survival and transformation
(g) *3q26; **~20%; ***cell proliferation and survival
*=chromosomal location
**=prevalence of mutation
***=function of gene product
^=identified in 50% without APC mutations

3 PRESENTATION AND MORPHOLOGY OF CRC

The majority of patients developing colorectal cancer will eventually present with symptoms. Main symptoms include persistent rectal bleeding, often with no anal symptoms, as well as change in bowel habit that persists over six weeks or more (tenesmus, frequent defecation or looser stools or both), abdominal pain and weight loss. Iron deficiency anemia caused by the bleeding from the tumor and signs of intestinal obstruction are secondary effects [87].

Symptoms can vary depending on the tumor site. Right-sided colon tumors often present late and have more general symptoms of fatigue, fever, weight loss, anemia and in 23% of cases have a palpable mass. Left-sided tumors tend to have more of cramping abdominal pain while fresh rectal bleeding and increased tenesmus is more common in rectal tumors [88].

It is important that primary care physicians who most often first see these patients do a physical examination including a digital rectal one as well as doing an occult fecal blood test and a full blood count to control for anemia. Rapid referral is then indicated to a suitable clinic that will continue the investigation with colonoscopy/rectoscopy, rigid proctoscopy and biopsy of the tumor.

Standard investigation for CRC also includes a Magnetic Resonance Imaging (MRI) of the pelvic area for rectal tumors, and for both colon and rectal tumors a computed tomography (CT)-scan of the thorax and abdomen. These are required for the radiological staging of the tumor to assess locally advanced disease and metastatic disease [87].

A full biochemical workup is done that includes besides blood count even liver- and kidney function tests as well as the tumor marker tests (CEA, CA 19-9, CA-125). Deranged levels of liver enzymes, high alkaline phosphatase (ALP) and lactase dehydrogenase (LDH) and low serum albumin imply advanced disease where the three latter tests also indicate poor prognosis. Elevated carcino-embryonic antigen (CEA), a specific CRC marker, is observed in approximately two-thirds of CRC cases and is used mainly with monitoring response to treatment and indicative of relapse [89]. Elevation of tumor markers CA 19-9, a marker of upper gastro-intestinal tumors and CA-125, a gynecological tumor marker and indicative of peritoneal metastases, can be seen in CRC but are not specific for it.

After the work-up is done the patients case is presented at a pre-therapeutic Multi-Disciplinary Team (MDT) conference. As part of the work-up before the MDT conference, information should be available if the patient has any comorbidity and if there is a family history of cancer.

The MDT-conference was established in clinics to discuss the individual patients case and recommend the best treatment option available for the patient. They occur weekly and are attended by oncologists, surgeons, radiologists, pathologists and contact-nurses. MDT conferences began primarily for rectal cancer cases but has now grown to a routine practice for all CRC cases as well as other malignancies. Although there are no randomized studies done to investigate the benefit of MDT conferences to the patient, reports have shown better results in rectal cancer outcome for patients whose cases were discussed at a MDT conference [90].

3.1 Primary Tumor Location and Pattern of Metastasis

About 20% of CRC develops in the cecum, 25% in the sigmoid colon, 10% in the rectosigmoid junction and 20% in the rectum [91].

Spreading of CRC can occur by lymphatic and hematogenous dissemination as well as contiguously and transperitoneally. Distant metastases are most often seen in liver, lungs, lymph nodes and peritoneum. For colon tumors and proximal rectal tumors, hematogenous dissemination is via the venous drainage of the intestinal tract to the portal system, and then to the liver, lungs, skeleton and at times other sites such as the brain. The venous drainage for the distal rectal tumors is through the inferior rectal vein into the inferior vena cava instead of the portal system which results in initial metastasis to the lungs.

3.2 Staging

The international tumor, node, metastasis (TNM) staging system introduced in 1959 is used for staging CRC and is continually updated by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). The most recent revision, is the eighth edition from 2017 [92].

The (T) in TNM stands for the invasiveness of the primary tumor, (N) is the number of regional lymph node metastases and (M) is the presence of distant metastases.

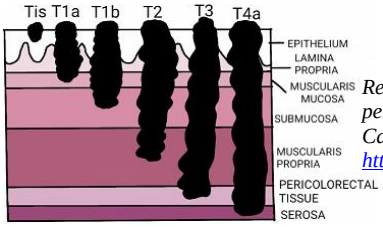
Updates in the latest TNM edition include a more clarified definition of pT4a, tumor deposit and distant metastasis. Factors which are important in treatment decisions but not yet incorporated into formal staging criteria are preoperative serum CEA, tumor regression score, lymphovascular and perineural invasion, microsatellite instability (MSI) and mutation status of KRAS, NRAS and BRAF.

The sixth edition of the AJCC staging system was used in study I-II [93], and the seventh edition in study III-IV [94]. The earlier pathological classification of CRC, Dukes staging system, can be translated to the current TNM staging system. See Table 3 for the different versions.

Table 3. TNM classification according to the 8th edition of the AJCC Cancer Staging Manual.

	TNM Stage *8th and 7th Edition				***TNM Stage 5th and6th Edition***			***Duke's***
Stage	T	N	M	***Stage***	T	N	M	
0	Tis	N0	M0	***0***	Tis	N0	M0	–
I	T1-2	N0	M0	***I***	T1-2	N0	M0	A
IIA	T3	N0	M0	***IIA***	T3	N0	M0	B
IIB	T4a	N0	M0	***IIB***	T4	N0	M0	B
IIC	T4b	N0	M0					B
IIIA	T1-2	N1/N1c	M0	***IIIA***	T1-2	N1	M0	C
	T1	N2a	M0					C
IIIB	T3-T4a	N1/N1c	M0	***IIIB***	T3-4	N1	M0	C
	T2-T3	N2a	M0					C
	T1-T2	N2b	M0					C
IIIC	T4a	N2a	M0	***IIIC***	Any T	N2	M0	C
	T3-T4a	N2b	M0					C
	T4b	N1-N2	M0					C
IVA	Any T	Any N	M1a	***IV***	Any T	Any N	M1	D
IVB	Any T	Any N	M1b					D
****IVC***	Any T	Any N	M1b					

Tumor-Node-Metastasis Classification

	AJCC 8th and 7th Edition	*AJCC 5th and 6th Edition*
	T (primary tumor)	
TX	Primary tumor cannot be assessed	Primary tumor cannot be assessed
T0	No evidence of primary tumor	No evidence of primary tumor
Tis	Carcinoma in situ: intra epithelial or	Carcinoma in situ: intra epithelial or
	Invasion of lamina propria	Invasion of lamina propria
T1	Tumor invades submucosa	Tumor invades submucosa
T2	Tumor invades muscularis propria	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericolorectal tissues	Tumor invades through the muscularis propria into pericolorectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
T4a	Tumor penetrates to the surface of the visceral peritoneum	 *Reprinted with permission from CancerBro: https://cancerbro.com*
T4b	Tumor directly invades or is adherent to other organs or structures	
	N (Regional lymphnodes)	
NX	Regional lymph nodes cannot be assessed	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases	No regional lymph node metastases
N1	Metastases in 1 to 3 regional lymph nodes	Metastases in 1 to 3 regional lymph nodes
N1a	Metastases in one regional lymph node	
N1b	Metastases in 2 to 3 regional lymph node	
N1c	Tumor deposit(s) in the subserosea, mesentery, or non peritonealized pericolic or perirectal tissues without regional nodal metastases	
N2	Metastases in 4 or more regional lymph nodes	Metastases in 4 or more regional lymph nodes
N2a	Metastases in 4-6 regional lymph nodes	
N2b	Metastases in 7 or more regional lymph nodes	
	M (Distant metastases)	
MX	Distant metastases cannot be assessed	Distant metastases cannot be assessed
M0	No distant metastases	No distant metastases
M1	Distant metastases	Distant metastases
M1a	Metastases combined to one organ or site (for example lung, liver, ovary, non-regional node)	
M1b	Metastases in more than one organ/site or the peritoneum	

Radiological imaging establishes if there is distant spread or metastasis and the histopathological staging which is made after surgical resection of the colorectal tumor determines any local and regional spread.

Other than the invasiveness of the primary tumor (T), the number of regional lymph node metastases (N) and the presence of distant metastases (M) the TNM staging system recommends other factors for routine assessment that aid in prognosis such as the presence of extratumoral tumor deposits, lymphovascular and perineural invasion, histologic grade of differentiation, preoperative level of CEA, MSI, RAS and BRAF mutations.

TNM is used in different settings where cTNM (c stands for clinical) refers to clinical findings and radiology, yTNM is used in assessing preoperative radiotherapy (RT) or

chemotherapy and pTNM (p stands for pathology) is the combined clinical, radiological and pathological assessment of the resected tumor.

An increase in T substage is correlated to a negative prognosis [95].

So as to not understage the tumor it is recommended by the AJCC and the College of American Pathologists that at least 12 lymph nodes are examined in the resected tumor specimen [92, 96]. The impact of lymph node status was supported by the large study done by the SEER database that found a 20% decrease in mortality when more than 15 lymph nodes were examined [97]. The higher the number of involved nodes, independent of the T category, correlates with a worse prognosis in stage III [97].

Residual tumor, defined as local residual disease after the completion of therapy, is an adverse prognostic factor [98].

Tumor deposits are defined as nodules of tumor within the pericolic, perirectal fat or in adjacent mesentery. They are often linked to extramural venous invasion and are equivalent to nodal metastases with their adverse prognostic influence [99].

3.3 Prognosis

Stage, which embodies the combination of TNM, is still considered the most significant determinant of the prognosis of the CRC as well as the management of it [100].

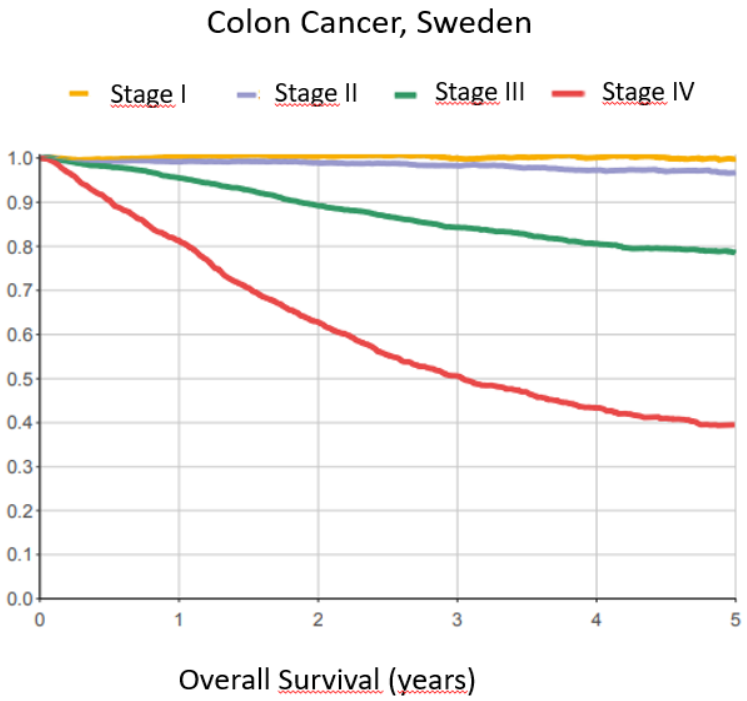
At diagnosis approximately 39% of CRC cases are localized or confined to the primary site, 35% have spread to regional lymph nodes, 22% have distant metastases and 4% have an unknown stage [101].

Generally, a more advanced stage correlates to a worse overall survival (OS), see Table 4 [102]. Figure 4 displays the relative overall survival curves for colon cancer and rectal cancer according to TNM stage as reported by the Swedish colorectal cancer registry [103].

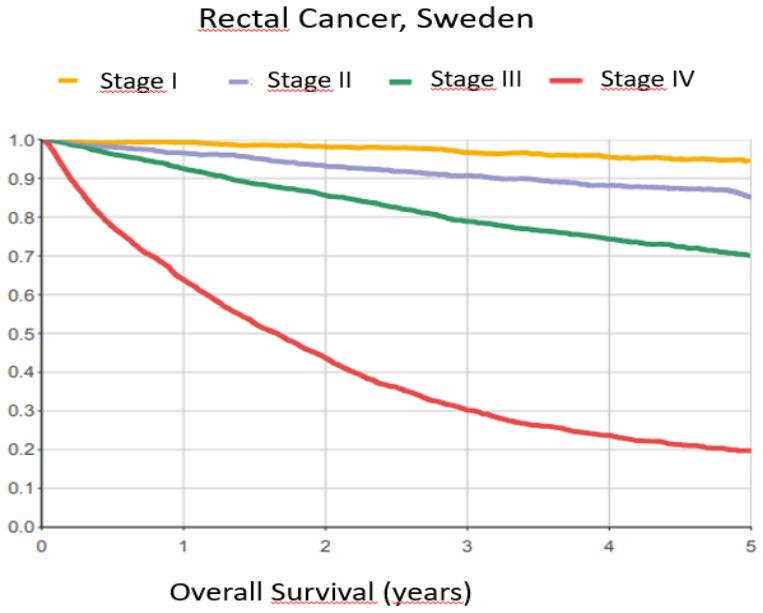
Table 4. 5-year relative survival rates for colon cancer and rectal cancer based on people diagnosed between 2009 and 2015

Stage	Colon Cancer	Rectal Cancer
Localized	90.4%	88.9%
Regional	71.3%	71.1%
Distant	13.8%	15.1%
All Stages combined	63.4%	66.7%
Unstaged/Unknown	26.2%	50.4%

*Based on data from the website of the National Cancer Institute (https://www.cancer.gov), accessed August 2019.



Relative Overall Survival, electively operated patients with colon cancer diagnosed 2012-2018.



Relative Overall Survival, operated patients with rectal cancer diagnosed 2012-2018.

Figure 4. Relative Overall Survival in colon cancer and rectal cancer in Sweden 2012-2018.

4 COLON CANCER - CURATIVE TREATMENT

Guidelines from the American Society of Clinical Oncology (ASCO), National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO) and National Swedish Guidelines aid in the recommendation of a suitable treatment for the patient which is then discussed with the individual patient.

4.1 Surgery

For localized colon cancer (stage I-III) surgery is the only curative modality. A successful surgical resection of the tumor involves complete removal of the tumor as well as of the major vascular pedicle and the regional lymphatic drainage basin. Depending on the site of the tumor surgical options are as follows: right hemicolectomy for tumors in the right colon; extended right hemicolectomy for tumors in the transverse colon; left hemicolectomy for tumors in the left colon and sigmoid colectomy for sigmoid colon tumors. A total abdominal colectomy with ileorectal anastomosis is reserved for selected patients with Lynch, FAP or metachronous tumors.

4.2 Chemotherapy Agents in Adjuvant Setting

Background on adjuvant chemotherapy

The goal of adjuvant (postoperative) chemotherapy is to eradicate micrometastases and thus reduce the risk of disease recurrence and increase the cure rate. The two chemotherapy agents used in the adjuvant setting are 5-Fluorouracil (5-FU), given intravenously combined leucovorin or as an oral pro-drug (Capecitabine), and Oxaliplatin. They are also used in metastatic CRC (mCRC) treatment.

4.2.1 5-Fluorouracil (5-FU)

5-FU, a fluoropyrimidine, is an antimetabolite drug developed in the 1950s, and is considered a cornerstone of both adjuvant and mCRC treatment. It causes cytotoxicity by misincorporation of fluoronucleotides into RNA and DNA as well as the inhibition of thymidylate synthase (TS), a nucleotide synthetic enzyme. More detailed information on thymidylate synthase is provided in section 7.3.

As 5-FU is an analog to uracil with a fluorine atom instead of hydrogen at the C-5 position, it can ‘quickly’ enter the cell where it is then intracellularly converted to the active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). FUTP is cytotoxic by damaging the normal RNA processing through its incorporation into RNA thus leading to faulty translation as well as obstructing synthesis of other forms of RNA (e.g. rRNA, tRNA, pre-mRNA). A major effect of 5-FU is by inhibition of TS by its metabolite FdUMP. FdUMP binds to TS instead of its usual substrate, dUMP [104].

The function of the enzyme TS is to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using the reduced folate 5,10-methylenetetrahydrofolate (CH_2THF). This important process supplies the only de novo source of thymidylate, one of the nucleotides necessary for DNA replication and repair. Lethal DNA damage by TS inhibition is then due to a depletion of dTMP and consequently of deoxythymidine triphosphate (dTTP). A further consequence of TS inhibiton by 5-FU is

the accumulation of dUMP leading in turn to increased levels of deoxyuridine triphosphate (dUTP). Both the 5-FU metabolite , FdUTP and dUTP can be misincorporated into DNA causing damage [105]. The enzyme thymidine kinase (TK) can salvage dTMP from thymidine thus providing a possible explanation of 5-FU resistance [104]. The mechanism of 5-FU is pictured in Figure 5 [104].

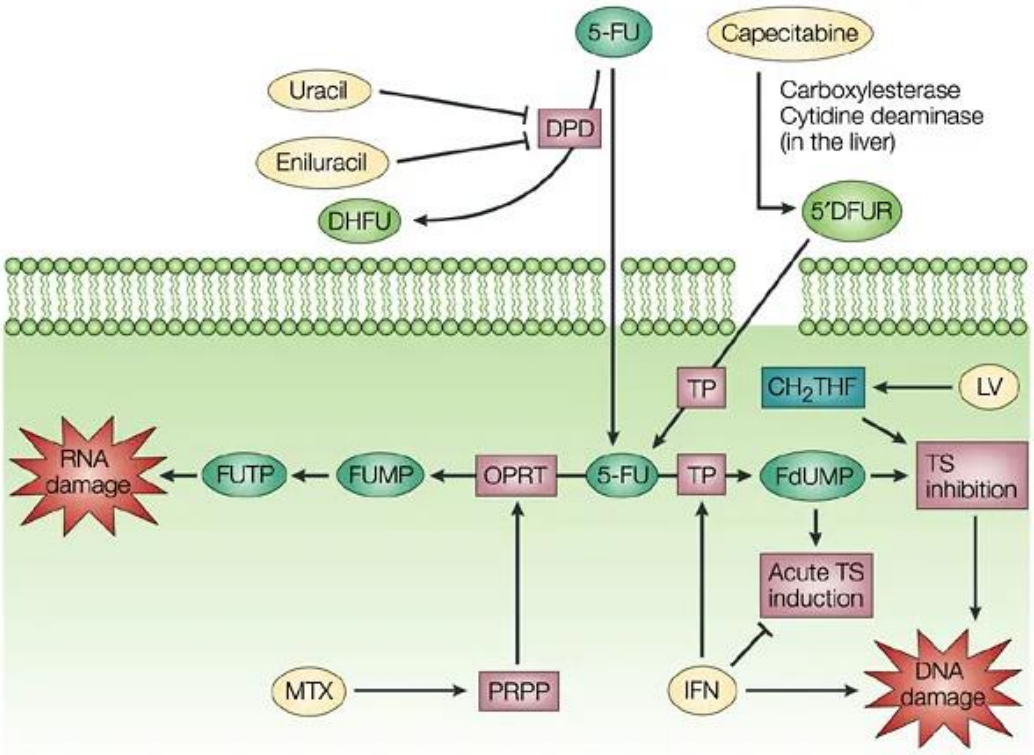


Figure 5. Modulation of 5-fluorouracil activity. *(Reprinted, with permission, from Longley et al. Nat Rev Cancer 2003)*

Leucovorin (LV) increases the intracellular pool of 5-10-methylene tetrahydrofolate (CH2THF), thus enhancing thymidylate synthase (TS) inhibition by fluorodeoxyuridine monophosphate (FdUMP). Eniluracil and uracil inhibit DPD-mediated degradation of 5-FU. Methotrexate (MTX) is thought to increase 5-FU activation by increasing phosphoribosyl pyrophosphate (PRRP) levels. Interferons (IFNs) have been reported to enhance thymidine phosphorylase (TP) activity, abrogate acute TS induction caused by 5-FU treatment and enhance 5-FU-mediated DNA damage. Capecitabine is a 5-FU pro-drug that is converted to 5´-deoxy-5-fluorouridiine (5'DFUR) in the liver by the sequential action of carboxylesterase and cytidine deaminase. 5'DFUR is converted to 5-FU by TP.

In an effort to improve the effect of 5-FU, different modulators have been developed. Leucovorin (LV) is an established modulator of 5-FU. Its function is to increase the intracellular concentration of CH2THF as well as stabilizing the ternary complex with TS and FdUMP thus in turn increasing 5-FU toxicity. Although it did not improve OS in mCRC, a meta-analys showed that the combination of 5-FU/LV had a better response rate (23%) compared to single 5-FU (11%) [106].

The oral fluoropyrimidine, the 5-FU prodrug Capecitabine, was designed to avoid dehydropyrimidine dehydrogenase (DPD)-mediated degradation in the liver. After absorption through the gastrointestinal wall it is converted in the liver to 5'deoxy-5-fluorouridine (5'DFUR) by carboxylesterase and cytidine deaminase. The enzyme thymidine phosphorylase (TP) that then converts 5'DFUR to 5-FU is more active in tumor

cells than normal cells which may contribute in clinical trials to capecitabine having a higher response rate (28.4%) than 5-FU/LV (15.5%), though survival was the same [107].

Another oral agent not commonly used in Europe and Sweden is UFT which combines uracil, a competitive inhibitor of DPD with tegafur, a 5-FU prodrug which is converted to 5-FU in the liver by the cytochrome P-450 enzyme.

4.2.1.1 Dehydropyrimidine dehydrogenase (DPD)

DPD, is the rate-limiting enzyme involved in 5-FU catabolism both in normal cells as in tumor cells. It is a rapid process and up to 80% of the degradation occurs in the liver [108]. The gene coding for DPD, DPYD, is located on chromosome 1, containing 23 exons in a single copy. A prevalence of 0.5% for partial or 5% for total deficiency of DPD has been identified as an autosomal recessive trait [109].

Approximately 30% of fluoropyrimidine treated patients will experience fluoropyrimidine-associated toxicity and 0.5-1% of them with fatal outcome [110]. It is estimated that 60-70% of cases of 5-FU toxicity are due to decreased levels of DPD. The most common allele variant associated with loss of DPD activity is DPYD*2A (c.1905+1G>A; rs3918290). It accounts for 25% of DPD-deficient patients. Genotype approaches to evaluate DPD deficiency vary from single nucleotide polymorphism (SNP) genotyping for evaluation of solely DPYD*2A or four validated DPYD variants or a full nucleotide analysis [111].

It has been shown in a clinical trial of >2000 patients that analysis of DPYD*2A and 5-FU dose reduction to approximately 50% for variant carriers, reduced grade 3 or higher toxicity from 73% to 28%, a rate similar to the *2A noncarriers. A disadvantage in the trial is that other DPYD polymorphisms were not identified. The analysis identified 18 as carriers of *2A and showed their screening process to be cost-efficient [112].

Other than SNP genotyping other methods used for measuring DPD deficiency are: measuring clearance of [2-13C]-labeled uracil in breath or serum level of uracil after an oral dose and enzymatic DPD activity in peripheral blood mononuclear cells [113].

ESMO guidelines have recommended DPD testing before 5-FU administration to be an option but is not routinely recommended [114]. The goal in the future is to increase patient safety as well as the clinical benefit by DPYD genotype-guided dosing.

4.2.2 Oxaliplatin

Oxaliplatin is a third generation platinum compound and has synergistic effects with 5-FU. It causes DNA damage by crosslinking the DNA double helix thus hindering DNA replication and transcription [115].

4.3 Adjuvant Chemotherapy Stage III

Stage III colon cancer patients are generally recommended adjuvant chemotherapy if no contraindications exist such as a significant level of co-morbidity and a poor performance status. After surgical resection the 5-year survival for stage III varies from approximately 30% to 60% [116].

The accepted timing for initiation of adjuvant chemotherapy within six to eight weeks after resection was adopted from the adjuvant colon cancer trials and supported by two meta-analysis [117]. Trials have shown benefit of adjuvant chemotherapy for the stage III

(node positive) colon cancer patients with a relative reduction of about 30% in risk of recurrence and 22 to 32% in mortality.

The backbone of adjuvant chemotherapy is 5-FU with the modulator, leucovorin (LV) [118]. The immodulatory drug, levamisole, is no longer used as a modulator to 5-FU due to its toxicity to the central nervous system.

Addition of Oxaliplatin to 5-FU/LV was shown in five randomized trials including the MOSAIC trial to increase the survival benefit but has the disadvantage of cumulative and dose-limiting peripheral neuropathy [119].

There are several studies, though no prospective randomized trials, indicating that dMMR or MSI-H colon cancers are resistant to 5-FU (fluoropyrimidine) chemotherapy [120-122].

Especially in stage III colon cancer patients with dMMR/MSI-H oxaliplatin retained a positive effect [123, 124].

4.3.1 Value of other oncological agents?

Three trials (CALGB 89803, PETACC-3 and ACCORD) have been negative regarding the value of adjuvant irinotecan-containing chemotherapy for resected colon cancer [125-127].

Targeted therapies with vascular endothelial growth factor (VEGF)-inhibition and epidermal growth factor receptor (EGFR)-inhibition have shown improved survival in the metastatic setting. They have however not shown benefit in the adjuvant situation. This may be due to the absence of a tumor burden in a micrometastatic setting and thus a difference in molecular biology. There is a need of predictive biomarkers for the adjuvant setting, especially in selecting patients which could benefit of targeted therapy.

The use of adjuvant bevacizumab (Avastin®), a monoclonal antibody inhibiting the vascular endothelial growth factor (VEGF), in addition to a 5-FU/LV adjuvant regimen did not improve the outcome of colon cancer patients in three large trials (NSABP C-08, AVANT trial, QUASAR2 trial) [128-130].

Lack of benefit in the adjuvant setting for colon cancer patients was also seen with the addition of an EGFR-inhibitor, cetuximab, to 5-FU/LV plus an oxaliplatin regimen (FOLFOX) in 2 trials (N1074 and European PETACC8 trial) [131, 132].

4.3.2 Duration of adjuvant treatment

Six-months of oxaliplatin-based chemotherapy is standard of care for the majority of patients with stage III colon cancer [133]. For adults older than 70 years, there is some evidence that there is little benefit to the addition of Oxaliplatin to 5-FU/LV [134].

There has been interest to shorten the duration of adjuvant chemotherapy. This is in part due to the cumulative and dose-limiting neuropathy of Oxaliplatin. In order to establish if shortening the adjuvant treatment would compromise the outcome, six trials have randomized between six versus three months of oxaliplatin-based adjuvant therapy. The trials results were summarized in a recent publication by the International Duration Evaluation of Adjuvant Chemotherapy (IDEA) Collaboration [135].

A suggestion is made from the results that high-risk cancers (T4 or N2) remain with the standard of a six-month treatment. However, due to only a small loss of absolute disease-free survival benefit (0.2% absolute difference at three years) and significantly lower Oxaliplatin neuropathy, they recommend a three-month treatment for low-risk disease (T1-3, N1).

4.4 Adjuvant Chemotherapy Stage II

The value of adjuvant chemotherapy for stage II (node-negative) colon cancer patients is controversial. Although surgery usually cures most stage II colon cancer patients, about 20-30% of patients will develop tumor recurrence and eventually die of metastatic disease [136]. No benefit of fluoropyrimidine-based adjuvant chemotherapy was seen for stage II colon cancer in four large trials [137-140]. In the largest of these trials, the QUASAR trial, 2146 stage II patients were randomized to adjuvant fluorouracil/LV with or without levamisole versus observation where they found a trend for better OS (Hazard Ratio (HR) 0.86, 95% Confidence Interval (CI) 0.54-1.19, five-year OS 83.9% versus 81.5%)) [138]. Three large trials (MOSAIC, NSABP C-07 and ACCENT) did not show any OS benefit with the addition of oxaliplatin to adjuvant fluorouracil/LV for stage II colon cancer patients [119, 141, 142]. Of interest, the MOSAIC trial found a sizeable trend towards a better disease-free survival (DFS) in high-risk (e.g. T4) stage II colon cancer patients who received an oxaliplatin plus short-term infusional 5-FU and LV (FOLFOX) [141].

Clinicopathologic features which are considered to contribute to the risk of disease recurrence in stage II colon cancer are: fewer than 12 nodes analyzed, T4 substage, perforated or obstructed tumor, poorly differentiated tumor, lymphatic, vascular or perineural invasion and MMR-status [116]. Adjuvant chemotherapy in stage II colon cancer is considered in the presence of one or more high-risk features.

In order for the patient to make an informed decision, the oncologist should discuss with the patient the relative risk of recurrence with or with adjuvant treatment as well as inform the patient of the expected side effects of chemotherapy.

Treatment guidelines require that MMR-status be analyzed for stage II CRC before treatment decision [143].

Studies have shown that MMR-status is a prognostic factor in Stage II colon cancer patients in which a dMMR or MSI-H status is correlated with a favorable prognosis [121]. Adjuvant chemotherapy is not recommended to patients with stage II CRC with no high-risk features and a dMMR/MSI-H tumor.

Although strong evidence is lacking, adjuvant therapy should be considered to patients with pMMR/MSS tumors and high-risk features. It is unclear what the best option is for patients with stage II dMMR/MSI-H and high-risk features. A reasonable option for these patients could be to give an oxaliplatin-containing regimen instead of fluoropyrimidene alone.

5 RECTAL CANCER – CURATIVE TREATMENT

An essential step for a correct assessment and treatment decision at the MDT-conference for a newly diagnosed rectal cancer patient is the pelvic MRI which provides the local radiological staging of the tumor.

5.1 Surgery

For the majority of stage I-III rectal cancer patients, radical surgical resection of the tumor is still the cornerstone of curative therapy. Advances in surgical techniques, especially the total mesorectal excision (TME) technique for low and mid rectum tumors, have decreased the local recurrence rate and overall survival of rectal cancer. With the earlier surgical technique of abdominoperineal excision (APE) local recurrence rates were close to 30% [144].

TME involves mobilization of the rectum, dissection in the avascular plane created by the mesorectal fascia (MRF) and removal of the tumor along with an intact mesorectum as well as less risk of tumor involvement in the lateral tumor margins. With TME-surgery local recurrence decreased to less than 10% [145, 146].

Patients that are fecal continent and have a rectal tumor in the low or mid rectum with no sphincter involvement most often undergo a low anterior resection (LAR) with the TME-dissection and bowel continuity. The abdominoperineal resection (APR) or abdominoperineal excision (APE) is subdivided into four options (intersphincteric, conventional, extra levator – ELAPE, and ischioanal) and is done in patients with lower-third rectal cancers in which there is involvement of the sphincters or pelvic fixation [147].

5.2 Radiotherapy (RT)

The role of RT with or without chemotherapy is established in the treatment of rectal cancer. Although the choice of oncological treatment for a rectal cancer regarding pre- or postoperative RT or chemoradiotherapy (CRT) and the benefit of chemotherapy has been controversial and differs between Europe and America.

Besides the radiological TNM staging, the location of the tumor determines the choice of treatment. The definition of a low tumor is up to 5 cm from the anal verge, middle from >5 to 10 cm, high from >10 up to 15 cm. The MRI can determine if there is any extramural vascular invasion (EMVI), the T substage and whether the mesorectal fascia (mrf) (equates to circumferential resection margin (crm)) is threatened and thus help predict which patients are at risk of local recurrence and distant metastases. This aids the mdt team define the necessary preoperative treatment and extent of surgery. Assessment of radiological lymph node staging is not always accurate. Upper rectal cancers (>12 cm from the anal verge, above the peritoneal reflection) are treated more like colon cancers.

A general categorization of the tumors can be done according to the Blomqvist and Glimelius recommendations in which ‘good’ tumors don’t require preoperative (C)RT and can go directly to surgery, ‘bad’ tumors generally require short-course RT (5Gy x5) and ‘ugly’ tumors require CRT [148], see Table 5. Guidelines [87] for preoperative treatment of rectal cancers are not absolute criteria for treatment. Treatment is also, for example, adjusted according to tumor location (anterior or posterior) and if tumor growth is <1mm to levator ani muscles.

Table 5. Categorization of rectal tumors based on radiological TNM staging.

Good	Bad*	Ugly
Low tumor: T1-2, N0, mrf-	Low tumor: T3, N1-2, mrf-	T4, T3 mrf+
High tumor: T1-T3b, N0, mrf-	High tumor: T3c-d, N1-2, mrf-	Lateral nodes (N+)

Mrf, mesorectal fascia; mrf-, unaffected mesorectal fascia; mrf+, affected mesorectal fascia.
Cut-off between a low and a high rectal tumor is around 8 (6-10) cm where the upper rectum is above the peritoneal reflection and the middle and lower rectum lies below it.
*if the tumor has signs of extramural vascular invasion (EMVI+) it is considered at least Bad

Contrary to colon cancer in which recurrence is mostly with distant metastases, rectal cancer's recurrence is equally distributed locally and in distant organs. RT of the rectum is more feasible and associated with less toxicity compared to the colon which is close to radiosensitive organs. The goal of RT is to lower local recurrence rate and aid in the improvement of survival together with a curative resection as well as to downsize and downstage locally advanced tumors allowing a radical (R0) resection. After different treatment schedules have been tried, the optimal RT dose for best effect has been set at 45-50 Gray (Gy) in 4-5 weeks (i.e. long course RT 1.8 Gy daily up to 28 fractions) or an equivalent dose using another schedule, such as short course RT, 5 Gy daily up to 5 fractions.

A Swedish trial in the late 1990s demonstrated a significant increased overall survival in rectal cancer patients that were randomized to preoperative RT (58%) versus direct surgery (48%), p=0.004 [149]. Other trials with the same randomization have shown a better decrease in local recurrence for those in the preoperative RT arm [150, 151].

With the improved TME surgery which in turn lead to a better prognosis of rectal cancer patients, preoperative (C)RT has shown in later studies to have an effect on local control but less impact on overall survival [151, 152].

The addition of chemotherapy to RT acts as a radio-sensitizer and is mainly used in locally advanced rectal cancers, i.e. long course RT 1.8 Gy to 28 fractions combined often with the oral fluoropyrimidine, Capecitabine. It is also theorized that the added chemotherapy may have an eventual early effect on occult metastases. Two randomized phase III trials comparing CRT to RT on locally advanced tumors (T3-4, N1-2) showed however only a decrease in local recurrence rate (11% to 5%) but no survival difference [153].

Three meta-analyses studies show that neo-adjuvant treatment (RT or CRT) is superior to adjuvant treatment (RT or CRT or chemotherapy) regarding reduction in local failure rates and cancer specific survival (CSS) [154-156]. Based on this evidence preoperative long-course CRT (total dose 50.4Gy with 1.8 Gy fractions concomitant with bolus 5-FU or Capecitabine) with a 6-8 week delay before surgery became a standard treatment for rectal cancer in Europe and Sweden [157].

The addition of 5-FU based chemotherapy to the postoperative RT showed in two trials a 10% significant increase in survival [158, 159].

Traditionally in North America, the decision for postoperative CRT was made after histopathological staging. Nowadays the NCCN guidelines recommend neoadjuvant CRT for transmural (T3/4) or node-positive tumors, especially for low rectal tumors [160]. Advantages of neoadjuvant treatment are: a better effect due to tumor proliferation being greater before surgery, increased tumor radiosensitivity, decreased tumor seeding and less toxicity which leads to eventually a better compliance. The disadvantage of risk of

overtreatment in patients with early stage disease has decreased due to better MRI imaging, a more correct radiological staging.

Several trials have explored whether neoadjuvant long course (C)RT versus short course RT on rectal tumors have similar effects on local recurrence survival, OS and toxicity. No significant difference between the two regimes was found regarding these endpoints in two trials, TTRAG trial and the Polish Colorectal Study group trial, which randomized T3 and T3/4 rectal tumors [161, 162].

RT technique has developed throughout the years leading to a more accurate treatment of the target, less radiation to risk organs and thereby decreased toxicity.

A complete pathologic response (pCR) of the resected rectal tumor is associated with a significantly lower recurrence rate and a better prognosis than with residual tumor, especially than with residual nodal disease [163].

5.2.1 Tumor regression grading system

Tumor regression grading (TRG) systems have been originally developed to categorize the amount of regressive changes in the histopathologic evaluation after (C)RT. Dworak et al demonstrated that the 5-year DFS rate was significantly correlated with the histopathologic tumor regression grade [164]. Dworaks TRG system as well as other TRG systems are illustrated in Table 6 [165]. The MRI-assessed tumor response (mrTRG) after (C)RT treatment in rectal cancer (Table 6) is also standard clinical practice as it is predictive of DFS and OS and provides prognostic information concerning the risk of local recurrence [166]. A mrTRG of complete tumor response is strongly correlated with a histopathologic complete response pCR [167].

Table 6. Tumor regression systems for rectal cancer

	Dworak	**AJCC**	**mrTRG**
Complete regression	No tumor cells (TRG 4)	No viable cancer cells (TRG 0)	Absence of any tumor signal (mrTRG1)
Near complete regression	Very few tumor cells (TRG 3)	Single or small groups of tumor cells (TRG 1: moderate response)	Small amounts of residual tumor visible but with a predominant fibrotic area of low signal intensity (mrTRG2)
Moderate regression	Dominantly fibrotic changes with few tumor cells or groups (TRG 2)	Residual cancer outgrown by fibrosis (TRG 2: minimal response)	Mixed areas of low-signal-intensity fibrosis and intermediate signal intensity present but with our predominance of tumor (mrTRG3)
Minimal regression	Dominant tumor mass with obvious fibrosis (TRG 1)	Minimal or no tumor cells killed (TRG 3: poor response)	Predominantly tumor signal intensity remaining, with minimal fibrotic low signal intensity (mrTRG4)
No regression	No regression (TRG 0)		No fibrosis evident, only tumor signal intensity visible (mrTRG5)

5.2.2 Complete response after neoadjuvant (C)RT

A meta-analysis showed an increase in pCR, 13.7% to 19.5%, and no significant complications when surgery was delayed more than the norm of 6-8 weeks after (C)RT treatment [168]. The finding that pCR seemed to increase if surgery was delayed after (C)RT lead to trials to explore whether timing of surgery was of importance.

One Swedish trial (Stockholm III trial) randomized rectal cancer patients: to short-course RT with immediate surgery, short-course RT with a 4-8 week delay before surgery and a long-course RT with 4-8 week delay before surgery [169]. They found that in comparing the short-course arms, delayed surgery patients had a higher rate of pCR (=TRG 4) compared to the immediate surgery patients, 11.8% vs 1.7%, $p<0.001$. Comparison of all arms showed no differences in rates of local recurrence, distant metastasis or OS.

The Stockholm III trial as well as others have led to a standard in Sweden of short-course RT with delay for the majority of rectal cancers and long-course CRT with delay for the locally advanced tumors. To avoid overtreating patients with RT and subjecting them to unnecessary side-effects from RT, the Swedish Guidelines for which rectal tumors should receive preoperative (C)RT are currently been revised.

If neoadjuvant therapy (C)RT achieves clinical and radiological complete response in localized rectal cancer, the approach of "watch-and-wait", thus achieving organ preservation and avoiding surgery, is showing to have safe and promising outcomes in several studies [170]. Close monitoring of these patients is required and further larger studies are needed before clinical implementation.

5.3 Adjuvant Chemotherapy

Evidence for adjuvant chemotherapy has not been as convincing for rectal cancers as it is for colon cancer. This is in part due to fewer rectal cancer patients participating in adjuvant trials to avoid the confounding factor of preoperative (C)RT that affects the tumor specimen histopathological assessment, ypTNM. Downgrading of the tumor stage by preoperative (C)RT is considered more of a prognostic marker than a predictive marker for response to postoperative adjuvant treatment.

Meta-analyses have shown some advantage of adjuvant 5-FU-based chemotherapy in DFS and OS but the analyses did not exclude studies that did not perform TME-surgery or use preoperative (C)RT [138, 171].

In a meta-analyses that included randomised trials of adjuvant 5-FU/LV proceeding (C)RT and surgery, no survival advantage was observed for adjuvant 5-FU [172].

Combining oxaliplatin to 5-FU/LV as adjuvant therapy for rectal cancer has not shown conclusive results on OS [173].

A better 'relapse-free survival' and OS was seen in high-risk rectal cancer patients receiving oxaliplatin plus 5-FU/LV that had no downstaging effect of preoperative CRT [174].

According to ESMO guidelines the oncologist should contemplate adjuvant chemotherapy in rectal cancer patients with yp stage III and high-risk yp stage II and have a risk-balanced discussion with the individual patient with regards to long-term effects of toxicity and risk for relapse [175].

In an effort to further establish whether rectal cancer patients would benefit with longer survival, a decreased risk for distant metastasis, a Swedish study (Rapido study) was done that tested neoadjuvant short-course RT followed by chemotherapy (6 cycles of capecitabine plus oxaliplatin, CAPOX) and then surgery compared to the standard arm of

long-course CRT followed by surgery followed by adjuvant chemotherapy [176]. The study has recently stopped including patients and the results are not yet published. Pending these results many clinics in Sweden have implemented the neoadjuvant concept of short course RT followed by chemotherapy (4 cycles CAPOX) as a study, LARCT-US (Locally Advanced Rectal Cancer Trial – Uppsala Style), that patients can be included into [177].

6 METASTATIC CRC

The mdt conference is of critical importance when establishing the potential goal of the treatment as well as the choice of a suitable type of treatment and its timing for a patient with metastatic CRC (mCRC), synchronous and/or oligometastatic (limited disease with few metastatic sites and lesions). Patients with metastatic disease are categorized into clearly resectable disease, initially unresectable disease but with conversion potential and unresectable disease.

6.1 Oligometastatic Scenario

In the stage IV, oligometastatic (OMD) scenario some patients, especially those with liver-limited disease can be surgically cured of their disease. Common metastatic sites include the liver, lung, peritoneum, lymph nodes and ovary. Metastases to the brain/central nervous system (CNS) or bones are correlated to poor prognosis and are usually not included into the OMD classification. Treatment strategy for OMD patients is a radical surgical resection of both the primary tumor and metastases. If surgery isn't possible, local ablative treatment (LAT) of the metastases can be an option.

In Sweden a common LAT for liver metastastases is the use of microwave ablation. Other forms of LAT are stereotactic ablative body radiotherapy (SBRT), a good option for lungmetastases, radiofrequency ablation (RFA), radioembolization (selective internal radiation therapy, SIRT) and external radiotherapy. A long-term survival has been observed in 20-50% of CRC patients with OMD that underwent R0 surgical resection of their metastases [178].

CRC patients with metastatic sites to the liver and especially the lung appear to have an improved prognosis, a longer survival time despite metastatic disease [179].

With radical surgical resection of lung metastases, 25-35% of selected patients reached a 5-year overall survival [180].

Although there is no international consensus and no prospective studies regarding the strategy for CRC patients with liver metastatases, ESMO guidelines has published recommendations [180]. They include the following 3 categories:

(1) Refers to patients with easily resectable liver metastases with excellent oncological prognostic criteria (e.g. limited number of liver lesions, long-term metachronous disease) where direct surgery is recommended. No perioperative chemotherapy is recommended here due to the negative results of the European Organization for Research and Treatment of Cancer (EPOC) trial with no 5-year OS advantage for the perioperative CT group compared to surgery-only group.

The benefit of adjuvant chemotherapy in this category is debated in which some evidence indicates benefit for patients with adverse prognostic factors and where ESMO recommends adjuvant FOLFOX or CAPOX if the patient has not recently (<6-12 months) received adjuvant chemotherapy.

(2) Recommends perioperative chemotherapy with 3 months preoperative (neoadjuvant) and 3 months postoperative FOLFOX or CAPOX regime, for easily resectable liver metastatases but only 'good' oncological prognostic criteria. Time duration and choice of chemotherapy regime is mainly derived from the EPOC trial.

(3) For easily resectable but 'bad' oncological prognostic criteria preoperative FOLFOX regime is recommended.

The use of bevacizumab, VEGF-targeting antibody, in category (1) and (2) is not recommended due to lack of data and no addition of EGFR-inhibitor is recommended for categories (1) and (3) based on data from the New EPOC trial [181].

In the third (3) category, addition of a monoclonal antibody would be accepted or triple chemotherapy, 5-FU/LV combined with oxaliplatin and irinotecan (FOLFOXIRI), plus or minus bevacizumab.

Synchronous metastases are seen in about 20-30% of newly diagnosed mCRC. In the cases with synchronous liver metastases the patient is usually recommended perioperative chemotherapy as in category (2) or (3), plus preoperative (C)RT for primary rectal cancer if needed, even if the liver metastatases is easily resectable and the patient has excellent or good oncologic criteria. Surgery is most often done in a two-stage resection with the resection of liver metastatases as the first stage and primary tumor resection as the second.

The liver surgeons present at the mdt conference have guidelines on what liver metastases are considered technically R0 resectable, a criteria also being at least a 30% future liver remnant must be maintained after resection [182].

The goal of conversion treatment with systemic therapy is to induce response in unresectable metastatic disease, often liver metastases, aiming to render them resectable. An increase in survival duration has been observed in patients who are then able to undergo resection compared to patients treated with CT alone. This, in spite the 75% rate of relapse later on, most often a hepatic recurrence [180].

There are several trials with different chemotherapy regimens that have been used in a conversion approach with liver-limited disease but few randomized, controlled trials to help identify the most effective regimen [183-189].

Based on these trials as well as other trials, ESMO guidelines and Swedish guidelines recommend as conversion therapy in RAS wild-type tumors a cytotoxic doublet plus an anti-EGFR antibody and in RAS-mutant disease a cytotoxic doublet plus bevacizumab or FOLFOXIRI plus bevacizumab [87, 114].

The majority of patients who respond to conversion therapy are thought to reach a maximal response to the treatment after 12-16 weeks and should thus be evaluated in time. Upon response, total therapy duration (pre- and post- surgery) is recommended to be no longer than 6 months.

The procedure of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) is done in CRC patients at specialized centers that have low-volume peritoneal metastasis, with a peritoneal cancer index (PCI) less than 20, and no or limited signs of systemic disease. Standard in Sweden for most of these cases is no preoperative chemotherapy but direct cytoreductive surgery, resection of the primary tumor as well as resection of peritoneal metastases and affected local ‘organs/ structures’ followed by intravenous bolus 5-FU/LV and then heated intraperitoneal oxaliplatin chemotherapy [87]. Post-operative adjuvant chemotherapy is based on the histopathological assessment, ypTNM and is often recommended if the patient is in good general condition.

6.2 Palliative Scenario

Palliative chemotherapy is applied when a patient with stage IV CRC is considered to have non-resectable disease. The majority (61%) of patients diagnosed with mCRC are in this category. The purpose of palliative chemotherapy is to prolong survival, alleviate symptoms and improve quality of life. There are several types of active drugs that are used in the treatment of mCRC.

6.2.1 Treatment drugs available

A major component in the treatment of mCRC is chemotherapy with fluoropyrimidines, parental (5-FU/LV) and oral (e.g. capecitabine), alone or in combination with oxaliplatin or/and irinotecan. Fluoropyrimidines and oxaliplatin are also used in the adjuvant setting as mentioned previously.

Irinotecan which is used in the metastatic setting is a topoisomerase I inhibitor and is usually used in combination with 5-FU/LV but also has effect as a single agent in mCRC [190, 191].

An oral agent, TAS-102 is also available. It contains a combination of active ingredients, trifluridine and tipiracil hydrochloride. Trifluridine is a nucleoside analog that acts as an antimetabolite that inhibits the enzyme thymidylate synthase as well as after modification in the tumor cell causes DNA strand breaks through incorporation into DNA. Tiparicil is antiangiogenic and a strong thymidine phosphorylase inhibitor thus inhibiting trifluridine metabolism.

Therapies targeting angiogenesis are:

- Bevacizumab (a humanized monoclonal antibody inhibiting the vascular endothelial growth factor (VEGF). Most commonly used clinically and for which there is substantial evidence for its use in first line treatment combination with chemotherapy and even in second line treatment.

Less commonly used in the clinical setting for refractory mCRC:

- Aflibercept, a fully-humanized recombinant fusion protein with VEGF binding parts from the human VEGF receptors 1 and 2 fused to the Fc part of human immunoglobulin G1.
- Ramucirumab (a humanized monoclonal antibody inhibiting the vascular endothelial growth factor receptor (VEGFR)).

Both, aflibercept and ramucirumab, can be used only in combination with FOLFIRI and can be considered in patients after progressing on an oxaliplatin-containing regimen and who received bevacizumab in the first-line treatment.

- Regorafenib is an oral active inhibitor of VEGF receptors 1 and 3 as well as stromal and oncogenic kinases.

TAS-102 and regorafenib can be considered for patients with refractory mCRC who still remain in a relatively good general condition and have been pre-treated with the available cytotoxics, anti-angiogenic, EGFR-antibodies. Both treatments have shown survival benefit where TAS-102 is less toxic while regorafenib requires dose adjustment and close monitoring due to its toxicity [192-195].

Therapies targeting the epidermal growth factor receptor are cetuximab and panitumumab. Cetuximab was first developed and is a chimeric human-murine monoclonal antibody which binds to the ligand-binding site of the epidermal growth factor receptor (EGFR) [196]. Panitumumab which entered the clinical setting in 2006, is an entirely human monoclonal antibody that also targets the EGFR but is much less associated with allergic and anaphylactic reactions compared to cetuximab. Both are main agents used in the clinic in mCRC patients whose tumors are RAS- and BRAF-status wildtype. They are considered

equally active as single agents and are used with chemotherapy-doublets, in combination with Irinotecan or as single agents [114].

Recently immunotherapies, nivolumab and pembrolizumab, which inhibit programmed death 1 (PD1) receptors have been presented as a possible treatment for dMMR/MSI-H mCRC .

6.2.2 Oncological treatment

Although the optimal manner to combine and sequence the agents has not been fully established, guidelines are continually evolving based on results of multiple trials and clinical experience. Generally, it is considered that the patient gets the best survival benefit if exposed to all agents during their treatment period rather than a specific sequence of administration. There are however factors to consider when choosing the first line treatment and beyond based on the patient's clinical condition (including age, performance status, co-morbidities), tumor burden, tumor location, tumor biology characteristics (RAS, BRAF and MMR-status) and treatment characteristics such as toxicity profile, flexibility.
The OS of mCRC patients receiving no palliative treatment, just best supportive care (BSC) is approximately 5-6 months.

Studies have shown that systemic fluorouracil (FU)-based chemotherapy alone improves progression-free survival (PFS) and OS compared to BSC alone [106, 197, 198].

Although there are no trials comparing BSC to combination chemotherapy of FU with oxaliplatin or with irinotecan, the increased survival of over 2 years has been proven in clinical trials using these combinations [198].

With the advent of biologic agents and the concept of an mdt-managed continuum of care, mCRC patients can attain a survival of approximately 30 months.

An mdt-managed continuum of care entails a 4-6 month 1st line induction therapy, upon response followed by possibly maintenance therapy and re-introduction of 1st line is possible upon progression or second-line treatment. 3rd line therapy is given upon progression of 2nd line and at times even a fourth line treatment thereafter or reintroduction of the 1st line therapy depending on the tumor biology and the patient's general condition.

6.2.3 First-line therapy

The choice of a first-line therapy (induction therapy) is individualized where patients that are symptomatic, have a large tumor burden and are in need of more aggressive therapy, receive at least a doublet plus a biologic or even a triplet if the patient is of younger age and fit. The aim is to hinder the rapid tumor progression. Most patients are asymptomatic where the aim is to have disease control and prolong survival.

As first-line FOLFOX, CAPOX or FOLFIRI are considered treatment regimens to have similar efficacy [199-204]. If the patient recently completed adjuvant chemotherapy with FOLFOX and was then diagnosed with unresectable mCRC the first-line therapy FOLFIRI is usually preferred. The combination of capecitabine and irinotecan (CAPIRI) is not used clinically due to its low level of evidence [205-207].

The triplet cytotoxic combination of FOLFOXIRI (5-FU/LV, oxaliplatin, irinotecan) has a superior effect to doublet chemotherapy but due to its increased toxicity is reserved predominantly for the younger patients with a good performance status and in need of tumor downstaging [203].

A known complication of oxaliplatin-based treatment is the cumulative neurotoxicity for which no antidote, as of yet, has been found. According to some studies, the majority of

patients that discontinue oxaliplatin-based treatment do so due to toxicity rather than progressive disease [208, 209].

The benefit of adding an antiangiogenic agents, e.g. bevacizumab to chemotherapy is greater when it's given up to tumor progression [208].

6.2.4 Epidermal growth factor receptor (EGFR)-inhibitor therapy

A review and meta-analysis of randomized, controlled trials involving EGFR antibody therapy found that mCRC patients with RAS wild type tumors had a significantly better OS compared to patients with RAS mutant tumors [210]. A negative prognostic effect was first demonstrated for the patients whose tumors harbored a mutation in the KRAS exon 2 (codons 12/13). Analysis of two mCRC trials with EGFR-inhibition, the PRIME study with FOLFOX4 +/- panitumumab and the CRYSTAL study with FOLFIRI +/- cetuximab, as well as other clinical similar trials found that mutations in KRAS exon 3 and 4 as well as NRAS exons 2, 3, 4 also predicted a negative response to EGFR-inhibition, especially when combined with a chemotherapy-doublet containing oxaliplatin [211, 212].

Even a BRAF analysis is a prerequisite before considering EGFR-inhibitors. A meta-analysis found that mCRC patients with BRAF mutant + RAS wild-type tumors had no significant added effect of EGFR-inhibition compared to standard chemotherapy alone [213].

Thus, a RAS mutation is considered a negative predictive marker in treatment with EGFR-inhibition and an expanded RAS analyses, plus BRAF analysis, is a requisite before considering this treatment in mCRC patients.

6.2.5 Treatment breaks

Trials exploring treatment breaks of palliative chemotherapy in mCRC patients have had varying results. A meta-analysis of eight randomized trials between continuous versus intermittent treatment, where 4 trials did not use maintenance therapy and 4 employed different types of maintenance therapy (fluoropyrimidine alone or with a biologic agent, two trials with biologic agent alone), did not show any disadvantage in OS or quality of life between the two arms, regardless of maintenance therapy [214].

A common guideline is that treatment breaks should not compromise outcome and must be individualized where the tolerance and response to the chemotherapy, tumor burden, symptomatology and tumor biology are taken into account. Tumors with a BRAF mutation have a worse prognosis, more aggressive disease in which treatment breaks are less appropriate.

6.2.6 Tumor location

Tumor location and its prognostic impact has been investigated in several trials. The right colon, extending from the cecum to the distal transverse colon, originates from the midgut while the left colon originates from the hindgut and consists of distal transverse colon to the rectum [215]. In the majority of studies, left-sided tumors are defined as tumors from the splenic flexure to the rectum and right-sided tumors as ones proximal to the splenic flexure [216]. About 65% of tumors occur on the left side and 35% on the right side [217].

Patient characteristics differ in that right-sided tumors are often more advanced tumors, poorly differentiated, mucinous and are more commonly seen in females and in older patients [218].

Molecular characteristics differ between the right and left colon in which right-sided tumors are often MSI-H or have dMMR-status, have a higher tendency to RAS and BRAF

mutations and a CIMP-H status while left-sided tumors are associated with the CIN pathway and the EGFR (or ErbB) signaling pathway [219].

Worse survival has been associated in right-sided tumors compared to left-sided tumors in metastatic CRC but not in stage II-III CRC [219-221].

A recent meta-analysis that analyzed tumor sidedness with regard to OS and PFS in 13 first-line clinical trials in mCRC found that the poor DFS in OS in right-sided tumors versus left-sided tumors was observed regardless of the chemotherapy regimen and the biologic agent [220].

A pooled analysis of six 1st-line randomized trials, total of 5760 mCRC cases, also confirmed a better prognosis in left-sided tumors compared to right-sided tumors, as well as a benefit for chemotherapy plus EGFR-inhibition versus chemotherapy alone or chemotherapy plus bevacizumab for left-sided RAS wild-type tumors [222].

These findings have prompted a 1st line treatment strategy based on tumor sidedness, see Figure 6. [223].

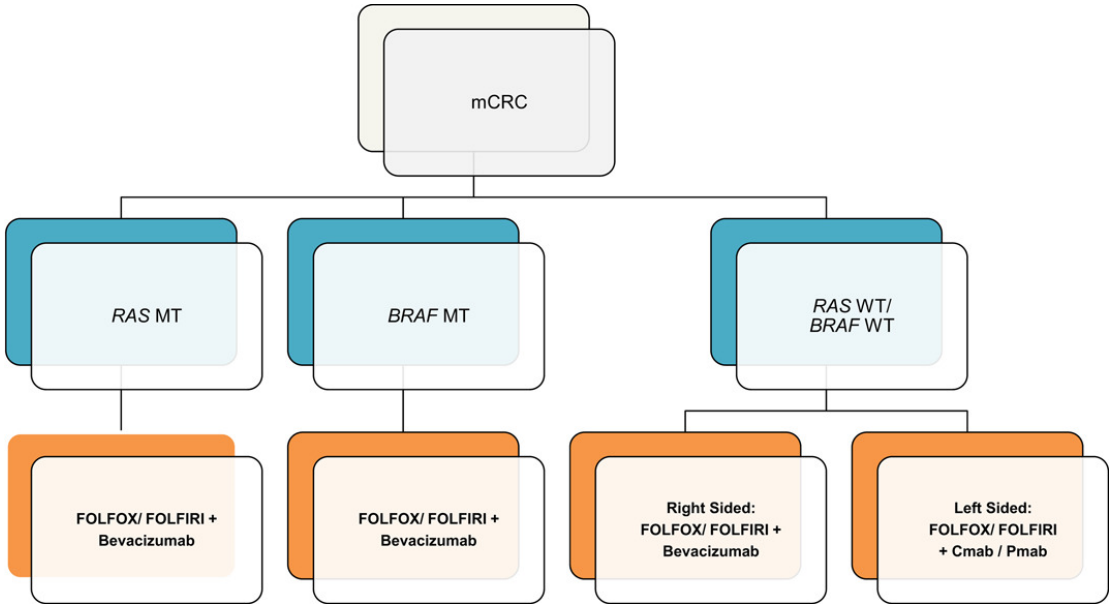


Figure 6. 1st-line treatment strategies in mCRC based on RAS/BRAF and sidedness.
(Reprinted, with permission, from Sandhu et al, J Surg Oncol 2019)

MT, mutated; WT, wild-type; Cmab, Cetuximab (EGFR-inhibitor); FOLFIRI, fluorouracil, leucovorin and irinotecan; FOLFOX, fluorouracil, leucovorin and oxaliplatin; Pmab, Panitumumab (EGFR-inhibitor)

6.2.7 Immunotherapy

Positive results from clinical trials that used the check point inhibitor, anti-PD1 therapy, in refractory dMMR or MSI-H mCRC has led the U.S. Food and drug Administration (FDA) to approve in May 2017, pembrolizumab ((Keytruda®) after progression to prior treatment with conventional chemotherapy and biologic agents [224].

Positive results have also been reported for another PD-1 inhibitor, nivolumab (Opdivo®), in patients with dMMR mCRC in a report from the Checkmate 142 phase II study [225]. In 2018, FDA approved the use of nivolumab (Opdivo®) plus ipilimumab (Yervoy®, a CTLA-4 inhibitor) in dMMR/MSI-H mCRC that has progressed after treatment with standard chemotherapy drugs.

Of interest will be the results from the phase III study, KEYNOTE-177 which randomizes pembrolizumab versus investigator choice of chemotherapy for dMMR/MSI-H first-line therapy in mCRC [226].

There are about 5% of mCRC that have a dMMR/MSI-H status and not all of them respond to checkpoint inhibitor treatment. The challenge remains in identifying further predictive markers of immunotherapy efficiency as well as finding newer agents /modalities alone or in combination with PD-1 inhibitors that may benefit these patients but also patients with pMMR/MSS tumors.

7 PROGNOSTIC / PREDICTIVE FACTORS IN CRC

Factors or biomarkers can be prognostic or predictive and at times both. A prognostic factor is a patient characteristic or a patients tumor characteristic that identifies subgroups of patients having different outcomes. It provides an estimation of disease outcome. A factor that predicts treatment effect, i.e. a predictive factor, is a patient characteristic or a tumor's characteristic that identifies subgroups of treated patients having different outcomes due to the treatment.

Validated and clinically established prognostic and/or predictive tissue-based factors in CRC are RAS-status, BRAF-status, MMR-status. They are in part discussed in the chapter pertaining to molecular pathogenesis of colorectal cancer as well as colorectal cancer treatment. They will be further discussed in this chapter.

Tumor location which is considered to have prognostic value and potentially a predictive value has been discussed in the mCRC treatment section.

This chapter will provide focus on the already established prognostic/predictive factor of MMR-status but also on the potential prognostic/predictive factors of TS protein expression, tumor budding, tumor border configuration and T-cell infiltration on primary CRC.

7.1 RAS, BRAF and Other Potential Factors

A critical pathway in the regulation as well as the proliferation and survival of cancer cells in CRC and other cancers is the mitogen activated protein kinase (MAPK) pathway. The pathway has an intracellular signalling cascade consisting of RAS, RAF, MAPK extracellular signal-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK1/2). It is known that activating mutations in RAS and RAF will activate this pathway [227].

KRAS mutations found in about 35-45% in CRC, occur primarily in codon 12 and 13, appear at a relatively early stage in tumorigenesis and are associated with tumors derived by the CIN pathway. Mutations in NRAS are found in about 4% of cases and in HRAS in about <1% of cases [227].

Implications of a RAS mutation in the treatment of a patient with mCRC have been discussed previously.

The BRAF mutation which occurs in about 10% in CRC, is associated with the MSI, CIMP and the serrated pathway. More than 80% of BRAF mutations are caused by a single mutation in nucleotide 1799 (T-A), known as BRAF-V600E, changing the amino acid from valine to glutamic acid in the BRAF protein. This results in a constitutive activation of BRAF [228].

As mentioned previously BRAF mutations in CRC are associated with a shorter PFS and OS.

The Epidermal Growth factor (EGFR) also known as ErbB-1 or HER1 belongs to a family of ErbB receptors, among then HER2/neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4).

EGFR-mediated signalling pathways include the RAS-BRAF-MEK-ERK1/2 (MAPK pathway), the PI3K-AKT-mTOR /PTEN as well as the JAK – STAT3-STAT3/STAT3 pathway. The insulin growth factor-1 receptor (IGF-1R) and the human epidermal growth factor receptor 2 (Her2) can also activate the MAPK pathway and the PI3K pathway.

Loss of phosphatase and tensin homolog (PTEN) reverses the activation of the PI3K pathway by dephosphorylating 3,4,5-triphosphate (PIP3) to 4,5-biphosphate (PIP2).

Mutations in PIK3CA are observed in CRC in approximately 8-9% of cases [227].

Unlike melanoma patients, the attempt to treat patients with mCRC and harboring a BRAF-V600E mutation with Braf-inhibitor was not successful. The mechanism behind this is believed to be the overactivation and crosstalk of the parallel pathway, PI3K-AKT-mTOR with the MAPK pathway. Even an EGFR activation can contribute to this [229].

Patients with RAS and BRAF mutated mCRC have restricted treatment options. There are no RAS inhibitors available for patients with RAS-mutated mCRC. For these patients and to some extent for patients with BRAF-mutated tumors, preclinical data supports dual target inhibition of MEK as well as one or more of the PI3K effectors as future treatment options for these patients.

An interesting finding in preclinical data is the activation of the Wnt pathway in KRAS and BRAF mutated tumors treated with MEK inhibitors. A possibility in the future could be combining inhibition of the Wnt pathway (i.e. with cyclosporine) with MEK1/2 inhibition [230]. Figure 7 illustrates the above named signaling pathways in CRC [230].

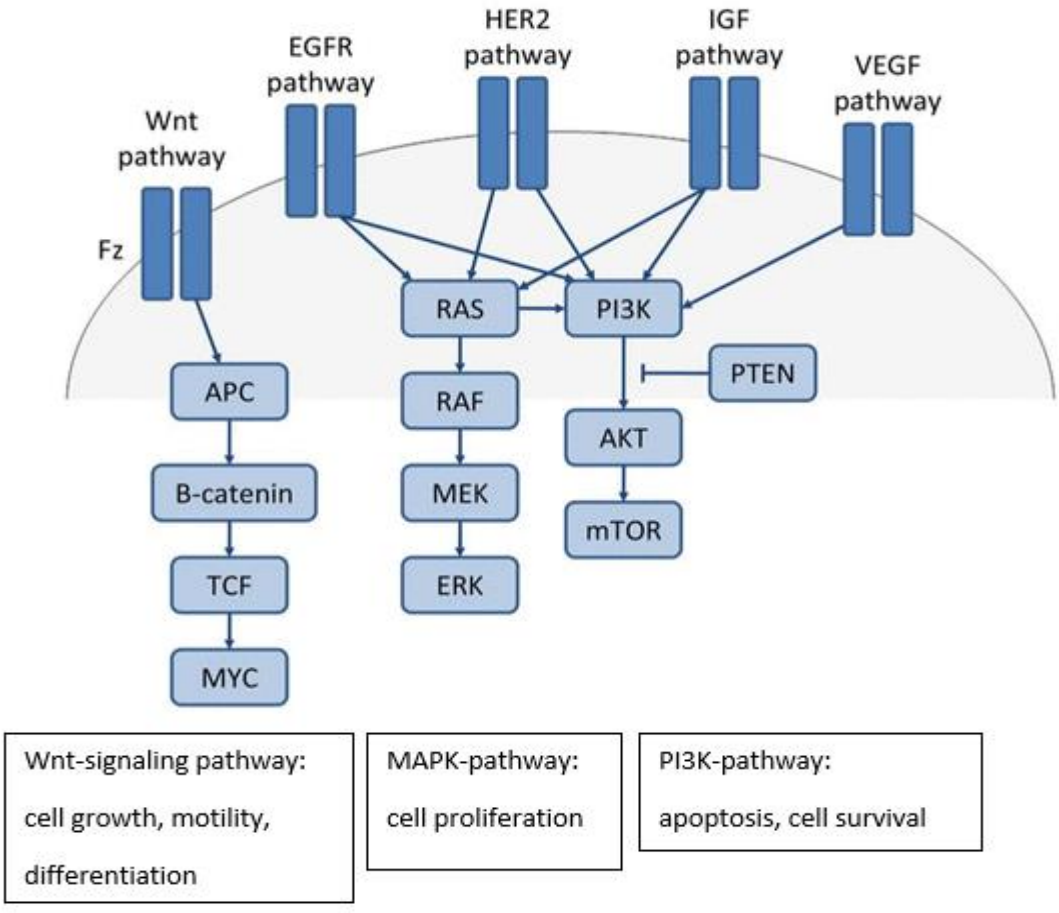


Figure 7._ Cell signaling pathways in colorectal cancer. *(Reprinted, with permission, from Moorcraft et al, Therap Adv Gastroenterol 2013)*

AKT, protein kinase B; APC, adenomatous polyposis coli; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; Fz, Frizzled receptor; HER2, human epidermal growth factor receptor 2; IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin; MEK, mitogen-activated protein kinase kinase; MYC, v-myc myelocytomatosis viral oncogene homolog (avian); PI3K, phosphoinositol 3 kinase; PTEN, phosphatase and tensin.

7.2 MMR

One of the best-defined biomarkers for primary colon cancer is DNA MMR status. The function of the MMR enzymatic process which recognizes and repairs DNA damage as well as the concept of MSI that results from MMR defect is described earlier in section 2.3. Close to 15% of CRCs, stage II and III, display MSI which is due to a defective MMR system most often caused by hypermethylation of the MLH1 promoter [231]. In cases of the Lynch syndrome it is caused by autosomal dominant constitutional mutations in DNA MMR. Identification of a dMMR is done by IHC as described in section 9.2 while MSI is diagnosed via PCR method (described briefly in section 2.3.1). Sporadic MSI-H/dMMR CRC is associated with an activating BRAF mutation while Lynch syndrome is associated with wildtype Braf status. In advanced CRC, stage IV, approximately 4-5% of tumors have dMMR/MSI-H status [231].

Focus on MMR status assessment is due to its role in Lynch syndrome detection as well as its prognostic role in CRC and its more debated predictive role of chemotherapy. More recently it has also gained predictive significance in advanced CRC where dMMR-status or MSI-H cancers have responded to immunotherapy with immune checkpoint inhibitor when compared to p-MMR status or MSS cancers.

The association of a higher density of T-cells in dMMR/MSI-H status CRC is believed to be explanatory to these findings [232, 233].

7.2.1 MMR -status as a prognostic factor

A better prognosis for dMMR CRC compared to pMMR has been demonstrated in several studies in which most used the PCR technique for MSI-assessment [234]. Studies evaluating MMR-status as a prognostic factor are heterogeneous with a blend of colon cancer to CRC, they are mostly retrospective studies, only a few are randomized trials and they use different chemotherapy regimens.

Stage II-III:

The first systematic review and meta-analysis from Popat et al, 2005 included 32 mostly retrospective studies with 7642 cases [121]. It included different stages (stage I-IV) in which the majority of the patients were from stage II-III. They found 1277 (16.7%) cases with MSI-H status and an overall improved OS was found for MSI-H compared to MSS (HR 0.65, 95% CI 0.59-0.71). No benefit from adjuvant 5-FU was found for patients with stage II MSI-H (HR 1.24, 95% CI 0.72-2.14). A second systematic review and meta-analysis from Guastadisegnia et al, 2010 included 31 studies with 12,782 cases where 1899 (14.9%) had MSI-H status [235]. Most studies were retrospective and seven of the studies were randomized. As in the Popat meta-analysis, they also found that MSI-H correlated with an improved DFS and OS and that MSS tumors had a significant beneficial effect of 5-FU therapy (OR 0.52, 95% CI 0.4-0.6, $p<0.001$).

A study from our group on MMR-expression on CRC stage II-III (randomized Nordic adjuvant trials) also found that a dMMR-status was an independent prognostic factor in sporadic CRC [236].

The prognostic effect of MSI was also retrospectively analyzed in 1404 cases, 210 of them with MSI-H (15%), included in the adjuvant PETACC-3 trial, stage II-III colon cancer randomized to 5-FU/LV alone or 5-FU/LV + irinotecan (FOLFIRI). MSI-H colon cancer was associated with a better relapse-free survival (RFS) and OS compared to MSS colon cancer [237]. The same patient material from the PETACC-3 trial was also later

analyzed by Klingbiel et al in 190 patients with MSI-H CRC and found a superior OS and RFS in stage II MSI-H CRC patients compared to MSS CRC patients [238]. Further analyses after adjustment for KRAS and BRAF v600E mutations showed the survival advantage for MSI-H versus MSS was for tumors located in the proximal colon but not in the distal location [239]. In their analysis the positive effect for patients with stage III MSI-H CRC seems to be limited to CRC in which a constitutional defect caused the MSI. Thus, it emphasizes to control for Lynch syndrome with germline analysis when needed.

In another retrospective analyses of randomized adjuvant trials (5-FU/LV +/- oxaliplatin) from NSABP with colon cancer stage II-III showed that dMMR was prognostic for recurrence compared to pMMR/MSS CRC patients but that MMR status was not predictive of oxaliplatin efficacy [240, 241]. Analysis of 986 patients from the MOSAIC adjuvant stage II-III colon cancer trial, randomized between 5-FU/LV alone versus FOLFOX found 95 (9.6%) patients with dMMR tumor status. A better DFS from FOLFOX compared to 5-FU/LV was found for a small number of cases with a dMMR status [242].

A more recent trial with 443 patients with dMMR colon cancer, randomizing 5-FU/LV versus FOLFOX in stage II-III CRC, found an improved DFS for FOLFOX only in stage III tumors and sporadic cases [124]. Retrospective analysis of 314 patients with MSI-H, KRAS-wild type stage III colon cancer in the NCCTG randomized CRC adjuvant trial between FOLFOX +/- cetuximab (anti-EGFR antibody) showed improved DFS in cases with tumors located in the proximal colon compared to the distal colon [243]. Adjustment was done in the analysis for prognostic factors including KRAS, BRAF mutations. The trial did not show a survival advantage for the addition of cetuximab [131].

Another recent study of 1250 patients with stage I-III colon cancer in which 138 had tumors with dMMR/MSI-H status, found MSI-H was associated with a reduced risk of nodal and distant metastases and a better DFS in stage I-II compared to stage I-II MSS [244]. In stage III, dMMR/MSI-H status was associated with worse pathological features and survival than pMMR/MSS .

7.2.2 MMR -status as a predictive factor

Stage II-III:

Ribic et al published in 2003 a retrospective analysis of 5 randomized trials comparing with or without adjuvant 5-FU chemotherapy in patients with MSI-H colon cancer (n=95) of the total of 570 cases [245]. They showed that 5-FU treatment benefited patients with stage II or stage III with MSS/MSI-L colon cancer but not patients with MSI-H colon cancer.

A large retrospective analysis supported this finding in which 253 patients with MSI-H CRC (mostly stage I-III with some stage IV cases) were found in the total of 1263 cases and no improvement of survival by adjuvant 5-FU treatment was found for MSI-H CRC patients [246]. The meta-analysis by Des Guetz et al with 6 studies of stage II-III CRC with a total of 3254 patients, of which 454 were MSI-H (14%) also found for MSI-H patients that there was no significant difference for RFS whether or not they received 5-FU chemotherapy [247]. A significant interaction between MSI status and therapeutic status was found which suggests a decreased 5-FU benefit for MSI-H compared to MMS status. Further corroboration to this finding was done by Sargent et al's retrospective analysis of 5 randomized clinical trials in stage II-III colon cancer between adjuvant 5-FU versus no adjuvant treatment [123]. For patients with MSI-H or dMMR tumors no benefit was found for adjuvant 5-FU in both stage II and stage III. A significant interaction was found between MMR status and treatment efficacy for DFS. However, authors from the studies

caution that this finding is to be interpreted with caution and to not omit standard 5-FU-based chemotherapy from stage III /MSI-H or dMMR CRC.

The first prospective trial showed that patients with CRC and dMMR status did not seem to benefit from 5-FU-based chemotherapy [248].

A study (QUASAR, multicentre trial) that did not show MMR-status to be a predictive marker, analyzed 1913 stage II-III CRC patients (218, 11.4% MSI-H) and the patients were randomized between adjuvant 5-FU or no adjuvant treatment. In their analysis, rate of recurrence (ROR) was significantly lower with adjuvant 5-FU than in the observation group. No marker, including MMR-, KRAS-, BRAF-status predicted benefit from adjuvant chemotherapy [249].

One systematic review and meta-analysis from 2015, analyzed 14 studies, 9312 patients with CRC (mostly stage II-III) in which 1398 had MSI-H status (15%) receiving single or mixed 5-FU-based regimens [250]. They concluded that the effect of 5-FU was not statistically significant for DFS or OS in MSI-H and therefore MSI was not a predictive marker. Included in this meta-analysis was the study from our group on MMR-expression on CRC stage II-III (randomized Nordic adjuvant trials) that did not find MMR-status to predict survival benefit from adjuvant 5-FU-based chemotherapy [236].

In conclusion, although there are some conflicting reports it is recommended to test MMR-status for patients with stage II CRC since they appear not to benefit from adjuvant 5-FU chemotherapy. The prognostic significance of a dMMR/MSI-H status in stage II CRC is more confirmed and accepted. It is still unresolved as to why the impact of an MSI-H status is different between stage II and stage III CRC and whether 5-FU chemotherapy is harmful in stage II dMMR/MSI-H CRC.

7.2.3 Role of MMR-status in mCRC

There are fewer studies on the role of MMR-status in mCRC. The better prognosis and assumed protective effect of a dMMR/MSI-H status in earlier stage CRC seems to diminish in advanced CRC [251].

One study explored the impact of BRAF mutation and dMMR/MSI-H status in mCRC in which of 524 patients 57 (11%) of them were BRAF-mutated [252]. The patients with BRAF mutated tumors had a poorer survival compared to BRAF wild type tumors as well as higher rates of peritoneal metastases and distant lymph node metastases and lower rates of lung metastases. In the study the patients with MSI-H tumors compared to the patients with MSS tumors had significantly poorer survival (11.1 months vs. 22.1 months, p=0.017). However, this difference was not observed in the BRAF mutant group. Thus, the poorer survival in mCRC seen in dMMR/MSI-H patients is most likely ‘associated’ with the negative effect of a BRAF mutation [253].

In 152 dMMR/MSI-H mCRC cases deriving from a pooled analyses of four phase III first-line treatment studies a worse DFS and OS was observed compared to pMMR/MSS mCRC [254].

An interesting observation is that recurrent CRC with a dMMR/MSI-H status has a lower response to conversion therapy. A theory is that chemoresistance may be due to the dMMR/MSI-H status [255].

7.2.4 Predictive role of dMMR/MSI-H for immunotherapy

A groundbreaking discovery for mCRC was the success of checkpoint inhibitors in dMMR/MSI-H. Thus MMR-status is now considered a predictive factor for immune therapy.

The improved prognosis in early stage dMMR/MSI-H CRC is thought to be due to the pronounced T cell infiltration. Defect in MMR causes hypermutations and increases the tumor neoantigen load, or the tumor mutational burden, which elicits an immune response.

In MSI-H mCRC it is believed that the process of adaptive resistance is triggered in which active T cells (Th1/CTL) and the tumor microenvironment cause a compensatory induction of checkpoints that protect the tumor from immune-mediated elimination [256].

More information on immune therapy is found in the section of immunotherapy 6.2.7.

7.3 Thymidylate Synthase (TS)

TS protein expression and mRNA levels in CRC have been the subject of research for many years due to its relationship to DNA synthesis and its role as a target of 5-FU.

TS is the rate-limiting target cytosolic enzyme of the necessary precursor of DNA synthesis (Figure 8) [257], 2'-deoxythymidine-'5'-monophosphate (dTMP) and the primary target of 5-FU, capecitabine, UFT as well pemetrexed and ralitrexed. The mechanism of 5-FU and the function of TS are reviewed in detail in section 4.2.1. As TS is a crucial part of the de novo source of dTMP, its inhibition results in the cessation of cellular proliferation and growth.

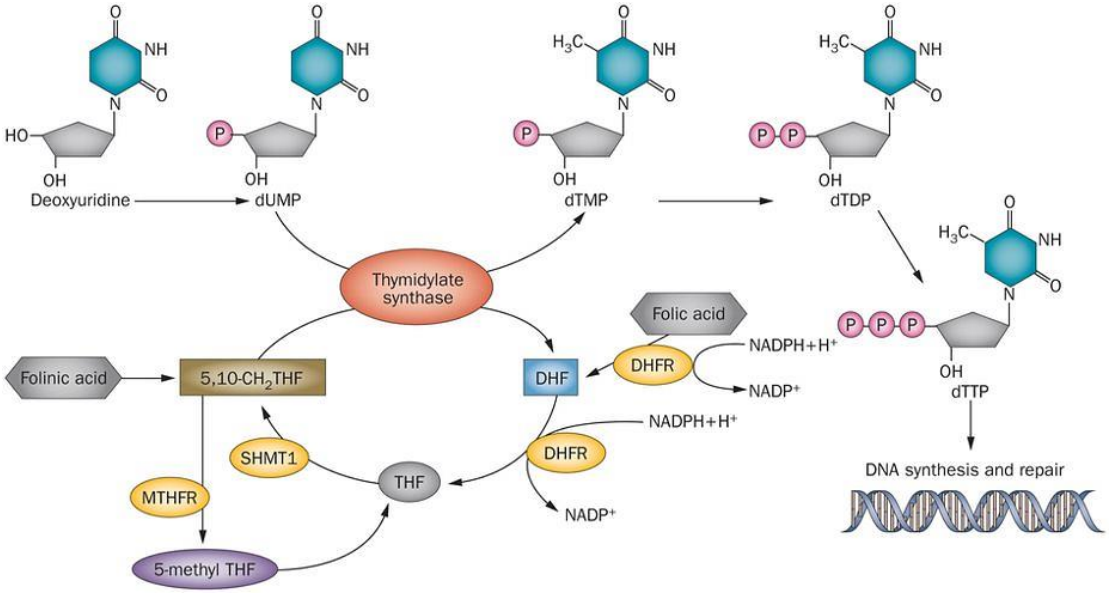


Figure 8. The thymidylate biosynthesis pathway. *(Reprinted, with permission, from Wilson et al, Nat Rev Clin Oncol 2014)*

TYMS, the human gene coding for the 36 kDa homodimeric TS protein, is located in the telomeric region on the short arm of chromosome 18, band 18p11.32 [258]. The TYMS gene contains a variable number of 28-base pair tandem repeats (VNTR) in the 5'untranslated region (UTR) that acts as an enhancer to the TYMS promoter [259]. It is proposed that three different polymorphisms in the UTR region act as modulators of TS mRNA transcriptional

and translational efficiency [260]. The majority of TS alleles carry either 2 or 3 VNTR repeats which define genotypes as 2R/2R, 2R/3R and 3R/3R [104].

About 25% of European patients are TYMS 3R/3R homozygous which increases TYMS expression and are less likely respond to 5-FU-based chemotherapy. The TYMS 2R/2R homozygous genotype, which decreases TYMS expression is seen in 20% of European patients [261].

An increase in 5-FU cytotoxicity may thus be caused by low TYMS expression, reducing the expression of the target TS and thus lowering the threshold to inhibit pyrimidine biosynthesis while a high TYMS expression may decrease the 5-FU cytotoxicity.

The QUASAR2 study in which 927 patients with CRC were treated with the oral 5-FU prodrug, capecitabine, showed that toxicity was associated with the TYMS polymorphism 2R/3R and 3'UTR6bp insertion-deletion and with the DPYD alleles 2846T>A and *2A [262].

There are no prospective trials studying whether pre-emptive TYMS genotyping is helpful in guiding fluoropyrimidine treatment management. Guidelines do not endorse TYMS genotyping and it is not performed in clinical practice.

TS transcription is regulated by several protein factors in which overexpression of E2F-1 seems to result in a TS mRNA and protein level increase [263]. The E2F gene family, in particular E2F-1, has a central role in regulating the transition in the cell cycle from G1 to S phase. TS is mostly active during the S-phase of the cell cycle [264]. The transcription factor, E2F-1, represses gene transcription when bound to the retinoblastoma (RB) protein as well as other corepressor molecules in nonmitotic cells. Upon loss of RB function, a constitutive induction occurs of E2F-1 genes that encode DNA synthesis enzymes, among them TS as well as DNA polymerase α and others. Rahman et al studied whether TS is a downstream effector of E2F-1 and thus might participate in the oncogenic pathway. They discovered that overexpression of TS resulted in neoplastic transformation in murine cells in vivo and in vitro and upon removal of serum containing survival factors, apoptotic cell death occurred [265].

Studies have shown that tumor cells have higher levels of TS mRNA than normal cells and this seems to correlate with a negative outcome [266].

Increased levels of TS protein levels as well as resistance to drugs targeting TS is caused by TS gene amplification, increased stability of the TS protein and translational derepression [267].

TS has been for several years, the subject of research as a potential prognostic and predictive marker in colorectal cancer.

7.3.1 Prognostic value of TS

Results from studies of the prognostic value of TS in the adjuvant CRC setting have been mixed. The majority of studies on mCRC have shown high TS levels to be associated with a poorer outcome [268].

A meta-analysis that included adjuvant as well as mCRC analyzed seven studies with regard to TS's prognostic value in the adjuvant setting (n=2610 patients) and thirteen studies (n=887 patients) in the metastatic setting [269]. All of the studies included were based on retrospective analysis and in 14 of the 20 studies treatment was given within a clinical trial. Smaller sample size was used in the metastatic studies with a median of 48 compared to the sample size of 184 in the adjuvant studies. In two of the adjuvant trials in which one of them is one of our previous studies, patients were randomized to either surgery alone or

surgery followed by adjuvant therapy [270, 271]. In one study, adjuvant treatment was given postoperatively to all patients [272] while in another study no postoperative adjuvant treatment was given [273]. The other 3 adjuvant studies were heterogeneous which included surgery alone, immunotherapy and chemoimmunotherapy [274-276].

All of the adjuvant studies in the meta-analysis used IHC as the TS evaluation method in which six studies used the TS106 monoclonal antibody as in our TS studies and one used a polyclonal antibody and an enzyme assay to evaluate TS [275]. In five of the seven adjuvant studies, TS expression was related to chromagen intensity and all adjuvant studies graded 0-2 as low and 3-4 as high levels of TS expression. One study [276] also graded high as grade 3 alone which we also did in our TS studies (Paper I, II) [277]. The median proportion of cases with high TS levels in the adjuvant setting was 50% (range 19%-77%) and was similar to the median proportion in the metastatic setting of 53% (range 14%-80%).

In the metastatic setting TS evaluation was more varied in which IHC was used in six cases, reverse transcriptase polymerase chain reaction (RT-PCR) in 5 cases, enzyme assay in one case and both IHC and enzyme assay in one case.

The conclusion of the meta-analysis was that tumors expressing high levels of TS seem to have a poorer OS compared to tumors with low levels where the combined HR for OS was 1.35 (95% CI, 1.07-1.80) in the adjuvant situation and 1.74 (95% CI: 1,34-2.26) in the metastatic situation. However, one has to consider that there may be publication bias, underpowered studies, heterogeneity with methodology and TS scoring between the studies.

A study done in our group by Öhrling et al, investigated whether TS expression in lymph node metastasis in stage III CRC was a prognostic marker [278]. In the entire study group (n=348) a significantly longer OS and DFS was correlated with low TS expression in the lymph node metastasis (multivariate, p=0.02 OS, p=0.04 DFS). In subgroup analysis, it was only a significant prognostic marker for patients in the surgery alone arm (OS p=0.04; DFS p=0.03) but not in the adjuvant chemotherapy arm. This could be due to adjuvant chemotherapy having a positive effect on tumors with high TS expression thus reducing DFS and OS differences between the arms.

A recent prospective analysis of TS as a biomarker for primary colon cancer showed that high tumor TS levels were associated with better DFS and OS compared to low levels [279]. Their study population was derived from two adjuvant studies, C89803 and C9581. The C89803 trial randomized 1,264 patients with stage III colon cancer to either postoperative adjuvant 5FU/LV or 5FU/LV plus irinotecan (IFL) [125]. The C9581 trial randomized 1,738 patients with stage II colon cancer to postoperative treatment with edrecolomab (an antibody inhibiting EpCAM) versus observation alone [280]. TS data was available for 435 (25%) patients with stage II and 463 patients (37%) with stage III. IHC using the monoclonal antibody TS106 was used for TS analysis and scoring with 0-1 as low and 2-3 as high. They also used an automated TS quantitative analysis (AQUA) for the C89803 cases. It measured TS localized in the nucleus, cytoplasm, sum of the two and ratio as continuous measurement. For all the 898 patients with TS available, 52% had high TS levels which is similar to the levels from other studies [269]. Substage analysis showed a significant difference with 44% high TS in stage III and 60% in stage II. In the entire group of 898 patients, patients with high TS expressing tumors had a DFS HR of 0.67, 95% CI (0.53-0.84) and OS HR of 0.68, 95% CI (0.53-0.88), log rank p=0005. TS was not significant in multivariable analysis of OS.

7.3.2 Predictive value of TS

The majority of studies have used IHC as the methods for assessment of TS expression.
There are conflicting results concerning the predictive value of TS in the CRC adjuvant setting.
Several studies, including Paper I in the thesis, have shown in primary CRC treated with adjuvant 5-FU chemotherapy that a high TS expression in the primary tumor seems predictive of a better outcome [275, 277, 281-287].

However, in five studies, in which the majority also assessed TS with IHC, no difference was found between low and high TS expression and their response to 5-FU adjuvant treatment [268, 270, 274, 276, 288]. In a study with neoadjuvant 5-FU therapy given to stage II-III rectal tumors, a better response was observed in patients whose tumors expressed low TS (analyzed using RT-PCR) [289]. Another study also showed a better response for adjuvant 5-FU in low TS expressing stage II-III CRC [290]. This study used in contrast to the other studies TMA instead of whole tissue assessment with IHC and could thus underestimate the presence of high TS expression in some areas of the tumor.

A recent prospective analysis mentioned earlier, with patients derived from two adjuvant trials, found that TS expression did not predict benefit of 5-FU based chemotherapy [279]. The predictive value of TS has been more convincing in studies done on mCRC.

In most mCRC studies in which the majority are retrospective, a low TS expression in the metastases has been associated with a better response to 5-FU [291-302]. Most studies tested TS in lesions from the primary tumor. One study that tested TS expression in the metastasis as well as the primary tumor found that low TS was predictive for response with assessment from the metastasis but not from the primary tumor [303].

Three studies did not find that TS expression in the primary tumor predicted response to 5-FU-based chemotherapy in advanced CRC [303-305].

One prospective study in mCRC patients (n=58) receiving either 5-FU/LV plus oxaliplatin or irinotecan, analyzed TS and DPD expression and did not find that low TS expression in the metastasis correlated with a better outcome [306].

In another prospective mCRC trial, tumor material (91 from liver lesions, 37 from the primary lesion) from 128 patients was analyzed for TS mRNA expression levels with reverse transcriptase polymerase chain reaction (RT-PCR) technique [307]. They found 82 patients (64%) had high TS and 46 patients (36%) had low TS. Patients in the low TS category as well as the high TS category were randomized to either 5-FU/folinic acid and irinotecan (FOLFIRI) or 5-FU/folinic acid (FUFA). They found a trend to a better overall response to FOLFIRI compared to FUFA in the TS high group (p=0.077). In the liver biopsy group with high TS expression, there was a significant better overall response for the FOLFIRI treatment (p=0.035). In the low TS group no difference in overall response was found between FOLFIRI and FUFA treatments.

The most recent prospective study, the phase II Eastern Cooperative Oncology Group (ECOG) E4203 trial, selected treatment for previously untreated mCRC patients based on tumor TS expression determined in mCRC tissue by IHC staining [308]. Of 186 eligible patients, 127 (68%) had high TS expression (score 2+ or higher) and 59 (32%) had low TS expression (score 0-1). The high TS group was randomized to either irinotecan and oxaliplatin plus bevacizumab (IROX/Bev) (n=61) or 5-FU/LV, leucovorin, and oxaliplatin plus bevacizumab (FOLFOX/Bev) (n=66) while the low TS group (n=59) received FOLFOX/Bev. The theory behind the treatment choice was that low TS tumors would respond better to a 5-FU/LV treatment combination than the high TS tumors. In summary,

TS expression was prognostic. Patients with low TS tumors receiving FOLFOX/Bev had a longer PFS and a trend to a longer OS, than patients with high TS tumors. No difference in benefit was seen between IROX/Bev and FOLFOX/Bev for the patients with high TS tumors.

7.4 Tumor Budding

Tumor budding is defined in literature as individual tumor cells or groups of up to four or five cells that are detached from the main tumor mass at the tumor invading front [309-311]. It is also known as peritumoral tumor budding and is observed in about 20-40% of CRC cases [312]. It was first recognized in the 1950s, described then as "sprouting" at the invasive edge of the tumor, and was thought to correlate with an increase in tumor growth rate. The term "tumor budding" was used first in a CRC study by Hase et al in 1993, in which they demonstrated a significant decrease in 5-year and 10-year survival rates with increasing budding [313]. Tumor budding was first used as a histopathological marker to estimate the aggressiveness of rectal cancer [309]. Studies and meta-analyses in CRC have demonstrated since then that tumor budding is an independent histopathological prognostic risk factor for a poorer outcome, especially in primary tumor stage II-III [311, 314-323].

7.4.1 Molecular background on tumor budding

Tumor buds are believed to be in a partial epithelial-mesenchymal transition (EMT)-like-state. EMT is a development regulatory 'program' that is thought to be the process by which transformed epithelial cells acquire a mesenchymal phenotype with the ability to invade, resist apoptosis and disseminate [324]. EMT involves epithelial cells losing cell to cell adhesion. A characteristic of mesenchymal cells is the increased expression of extracellular proteases and transcription factors (e.g. snail, slug, twist) that in turn stimulate cells to produce collagen, fibronectin, vimentin, α-smooth muscle actin [325]. Degradation of the extracellular matrix by metalloproteases and disruption of the basement membrane allows cancer cells to move along the matrix. A conversion from an epithelial to a mesenchymal state is complex with several steps and intermediate phenotypes. A 'hallmark' of EMT is the loss of the membranous localization of the adherens junction molecule, E-cadherin [326]. E-cadherin has been reported as a potential biomarker for colorectal cancer [327]. β-catenin interacts with E-cadherin at the adherens of epithelia. The loss of E-cadherin interaction with β-catenin is thought to result in nuclear translocation of β-catenin and activation of the Wnt signaling pathway, a process seen in the CIN-pathway of carcinogenesis [325], (see section 7.4.1, Figure 2). Disturbance of E-cadherin expression as well as the loss of cell membrane B-catenin expression is commonly observed in tumor buds [325].

Thus, a dysregulation of the Wnt-pathway contributes to cancer progression by disruption of cell adhesion and migration. The Wnt signaling pathway also has a role in the initial activation of stem cells, stem cell maintenance as well as crypt homeostasis [328]. A future therapy in CRC could be one targeting the Wnt-pathway.

Tumor buds are linked to partial-EMT and not a complete-EMT profile since they express some EMT-related markers but their association with increased levels of mesenchymal markers is not conclusive [325].

7.4.2 Tumor budding methodology

Tumor budding can be underestimated due to factors such as active fibroblasts in the desmoplastic stroma that liken tumor buds as well as dense inflammatory infiltrate that can obscure tumor budding cells [329, 330].

To avoid this underestimation many studies on tumor budding in CRC have used pan-cytokeratin IHC to highlight the tumor buds [329, 331-334].

Studies have shown that tumor budding is underestimated when using hematoxylin and eosin (H&E) compared to IHC [329, 335].

Prall et al, showed in stage I-II CRC that IHC with MNF116 discovered more high-grade tumor budding (59/186) than H&E (26/186) [329].

There has been a lack of standardization with regard to the field-of-view depending on the microscope used as well as specifying the area (mm^2) analyzed. Choice of tumor field has varied from overall tumor, entire advancing edge and field of maximum tumor budding.

Different approaches have been used as to whether tumor budding should be categorized subjectively or qualitatively, semi-quantitatively or with a specific count and cut-off. The cut-off of what should be considered high-grade tumor budding versus low-grade tumor budding has been varying and acquired with different methods. Figure 9 illustrates the different approaches [336].

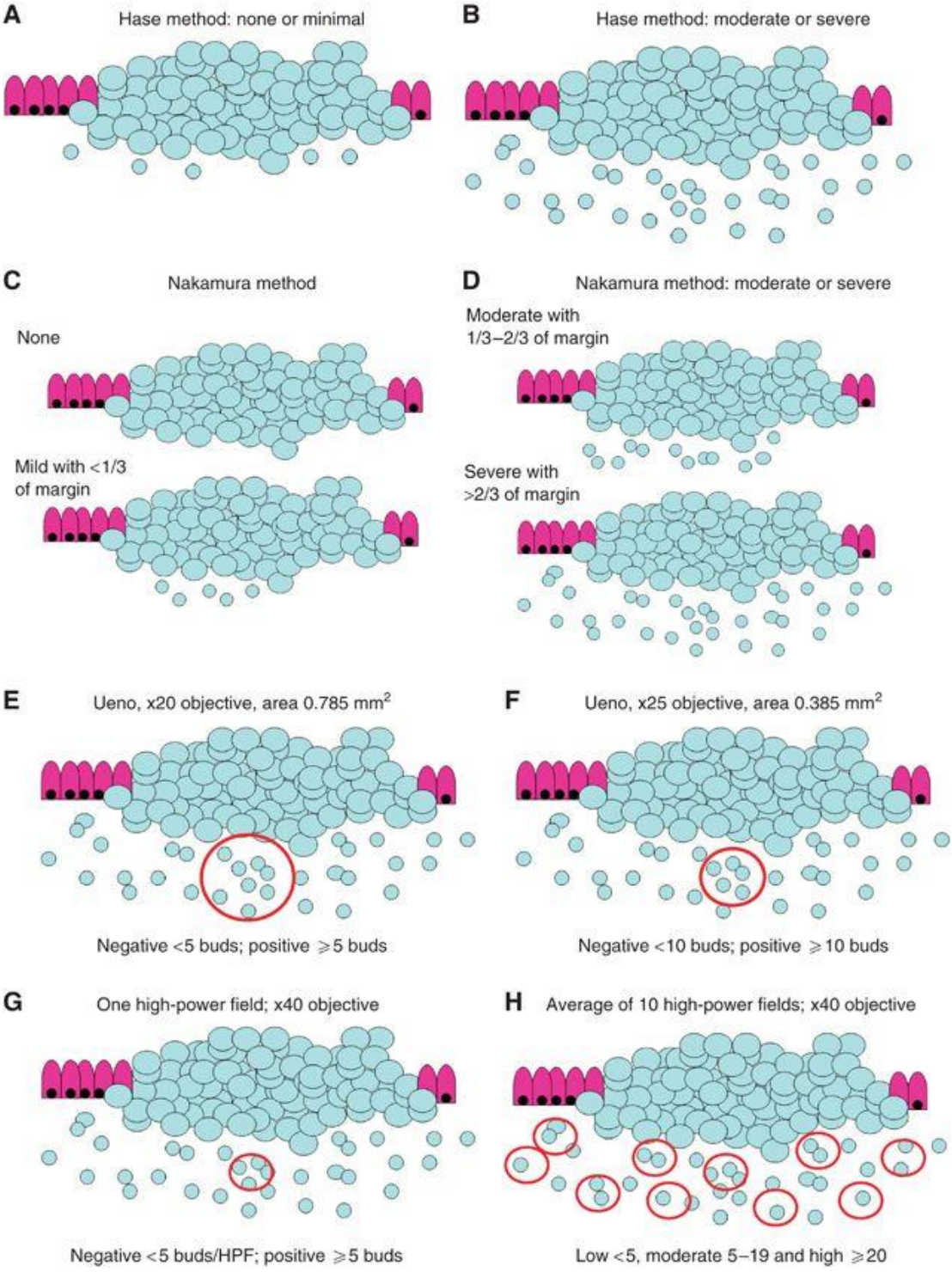


Figure 9. Visualisation of proposed tumor budding scoring systems. *(Reprinted, with permission, from Lugli et al, British journal of cancer 2012).*

(A,B) According to Hase et al, 1993 [313]
(C,D) According to Nakamura et al, 2005 [337]
(E,F) According to Ueno et al, 2002 [309]
(G) Recommended by Lugli et al, one high-power field average [336]
(H) Recommended by Lugli et al 2012 [336]

Puppa et al, 2012 studied the reproducibility of five tumor budding assessment methods among 10 investigators, using H&E and AE1-3 cytokeratin stained whole-slide digital scans from 50 pT1-T4 CRC [338]. They found in general a fair level of diagnostic agreement for tumor budding that was significantly higher in early cancer as well as among experienced gastrointestinal pathologists. Interestingly, cytokeratin IHC did facilitate detection of tumor buds but it did not affect interobserver agreement.

A review by Zlobec, 2013 [339] as well as a review by De Smedt, 2016 [312] recommends using a 10-HPF method on pan-cytokeratin-stained sections established by Karamitopolou et al, 2013 [340] as this method had the best inter-observer agreement. This method used the cut-off 10 tumor budding cells determined by receiver operating characteristic curve analysis with OS as end-point [340]. Paper IV in the thesis has adapted this method for tumor budding assessment.

Irrespective of which scoring system is used to quantify tumor budding, studies have shown that the more tumor buds identified by histological evaluation in CRC, the worse the prognosis [311]. The potential clinical value of tumor budding assessment is hindered by the lack of its universal implementation in diagnostic practice. This is due in large part due to the absence of standardization of tumor budding evaluation. However, the International Tumor Budding Consensus Conference (ITBCC) held in 2016 has reached a consensus that is evidence-based with a standardized scoring system for tumor budding in routine practice [341]. The guidelines recommend using H&E slides and analysis of tumor budding in one hotspot field with a specific size of 0.785mm^2. Classification of tumor budding is then done into three categories with low budding defined as 0-4 buds, intermediate budding (5-9 buds) and high budding (≥10 buds).

The recent AJCC 8th edition as well as the College of American Pathologists guidelines now include tumor budding as an optional reporting field [342].

7.4.3 Tumor budding in malignant polyps, preoperative biopsy

Multiple studies have shown that in malignant colorectal polyps (pT1) tumor budding is associated with an elevated risk to develop lymph node metastases [343-346].

Two studies showed a significant decrease in survival when high grade tumor budding was present in pT1 CRC [309, 313].

Tumor budding can also be present within the tumor and is referred to as intratumoral tumor budding. It is used especially in preoperative biopsies and has shown that tumor budding is associated with the presence of lymph node metastasis, a higher tumor grade, lymphovascular invasion, in the resected specimen and distant metastasis [344, 347-350].

Poor response to neoadjuvant therapy in rectal tumors was observed for patients whose preoperative biopsy showed presence of intratumoral tumor budding [347].

Due to these findings the National Comprehensive Cancer Network (NCCN), version 2.2017 has added tumor budding as something to be considered in clinical treatment and cautions against polypectomy as an adequate treatment for pT1 malignant polyps with signs of tumor budding [351].

7.4.4 Tumor budding stage I-II

An association was observed in resected stage I CRC, pT1/2 N0 M0 between high grade tumor budding and an increase in risk for lymph node metastasis [343, 346, 352, 353].

A main research focus is on tumor budding and stage II CRC in order to determine if high-grade tumor budding should be a factor to be considered in treatment decision of

adjuvant chemotherapy. Stage II CRC is a diverse group with differing survival in which survival can differ from stage IIA (T3N0M0) in colon cancer with 66.5% to rectal cancer stage IIC (T4bN0M0) 32.3% and a worse survival for colon cancer stage IIb (T4aN0M0) compared to stage IIIa (T1-T2 N1/N1cM0) [342, 354].

Several studies despite different methods of tumor budding assessment and scoring have shown that tumor budding in stage II CRC is an adverse prognostic factor, associated with poor DFS and OS [313, 329, 355-361].

One of the studies by Koelzer et al had a prospective cohort that also determined high grade tumor budding to be a negative factor for survival in stage II [358].

7.4.5 Tumor budding stage II-III

Several studies combine stage II-III CRC or at times stage I-IV in their analysis of tumor budding. While tumor budding is regarded as a marker of tumor progression even in more advanced (stage III) and metastatic CRC (stage IV), its role in clinical practice has to be further investigated.

One study that explored tumor budding solely in stage III CRC with 447 patients, using H&E slides and a x200 field of densest budding and a cut-off of ≥9 buds as high-grade tumor budding, found no association between tumor budding and survival outcome [362]. A study done recently with stage III colon cancer (n= 150) and using the recommended ITBCC guidelines for tumor budding assessment found tumor budding to be an independent variable that predicted recurrence [363].

Two studies on rectal cancer, both using H&E slides and densest tumor budding area in x200 field, found high grade tumor budding to be associated with decreased OS [309, 364]. One of the studies included stage I-III (n=638) and had a cut-off of 10 [309] while the other included stage II-III (n=90) and had a cut-off of >25 [364].

A study including stage I-IV CRC (n=381) using H&E slides, a x200 field and cut-off of 10 found tumor budding to be associated with an unfavorable outcome in cancers with mucinous differentiation [365].

Another study prospectively including stage I-IV CRC (n=299) and pan-cytokeratin staining slides with a x400 field found high grade tumor budding to limit PFS [366].

One study of Karamitopoulou et al, also using stage I-IV CRC (n=215) and pan-cytokeratin stained slides as well as the 10-HPF method in x400 field with a cut-off of 10, showed good prognostic relevance for tumor budding [340]. Paper IV in the thesis has implemented the tumor budding assessment method from this study. A larger study with 553 patients with CRC, stage I-IV, using H&E slides in a x200 field with a cut-off of 10 buds also confirmed a prognostic significance for tumor budding [367].

7.4.6 Tumor budding in mCRC

Tumor budding has not been extensively studied in mCRC. One study determined that tumor budding was associated with poor response to EGFR-inhibition therapy in mCRC [368]. Presence of tumor budding in CRC was a prognostic factor for lung metastases [337].

7.5 Tumor Border Configuration

The histomorphological feature of tumor border configuration or the growth pattern at the invasive margin in CRC, should not be confused with tumor budding in which tumor

budding is diagnosed at high magnification while tumor border configuration is more easily diagnosed, even on standard H&E slides, at a lower magnification [369].

An infiltrative growth pattern is characterized by tumor tissue disrupting the anatomic structures of the bowel wall and has little or no desmoplastic stromal reaction, including the absence of T-lymphocytes [370, 371]. The expansive or pushing growth pattern displays a distinct boundary between tumor and host tissue where a desmoplastic stromal reaction with the presence of T-lymphocytes is more commonly observed [370, 372, 373].

Classification of tumor border configuration has been described as either infiltrative or pushing/expansive by Jass et al [374] or as a three-tier system of infiltrative, intermediate or expansive by Morikawa et al [375]. An invasive tumor border configuration can be focal and not involve the complete tumor border which leads to variability of interpretation. In study IV we chose to analyze tumor border configuration by the Morikawa method in which the tumors classified as infiltrative had an unambiguous infiltrative growth pattern throughout the entire tumor border. In order to establish tumor border configuration in daily diagnostic practice, a more standardized consensus of its quantification is required.

An infiltrative tumor border configuration in CRC is considered a stage-independent negative prognostic indicator indicating an aggressive tumor with a higher risk for nodal and distant metastasis [371, 375-380].

Studies in CRC have shown that tumors with an infiltrative tumor border configuration often have high grade tumor budding which suggests an inter-relation in their biological formation [379, 381].

Association of an infiltrative tumor border configuration and other adverse molecular alterations / clinicopathological features including BRAFV600E mutation [375] and vascular invasion has also been described [379, 380].

The pMMR status in CRC is associated with an infiltrative tumor border configuration while the expansive one is associated with a dMMR status, especially in Lynch tumors [372, 373, 382].

A study by Zlobec et al on stage II-III CRC with pMMR-status showed that an infiltrative tumor border configuration was associated with a higher local recurrence compared to an expansive tumor border configuration independent of CD8+ TILs and lymph node status [378]. Morikawa et al showed that tumor border configuration was an independent prognostic factor independent of peritumoral lymphocytic infiltration [375].

To further identify patients with high risk of recurrence and optimize a therapy approach, the AJCC recommends assessment of tumor border configuration for transmural invasive CRCs, especially stage II tumors. Due to inconclusive data it is not yet recommended for early stage CRC, stage I [369].

7.6 T-lymphocyte Infiltration in CRC

7.6.1 Background on immune system

The immune system plays an important role in preventing occurrence of cancer. The immune host response has been of interest especially in terms of tumor infiltrating immune cells [383, 384].

Histopathological reports confirm that colorectal cancer tissue is invaded by immune cells from the host. The immune microenvironment and the immune response towards the tumor is created when the CRC neoplasia invades through the muscularis mucosa into the

submucosa causing a local host reaction with the accumulation of proinflammatory cells along the tumor margin [385].

The immune system is said to be an effective "gate-keeper" against cancer in normal conditions. The initial antitumor activity is mediated by the innate immune system entailing effector cells like Natural Killer cells (NK), neutrophils and macrophages. Thereafter a specific adaptive immunity is activated generating memory cells, B and T cells. T cells can, in general, be classified as helper T cells (Th cells, CD4+) or cytotoxic T cells (Tc cells, CD8+). Of special importance is the differentiation of naïve CD4+ Th cells into Th1 cells that produce interferon gamma (IFN-γ), promoting CD8+ T cell-mediated adaptive immunity. The presentation of tumor antigens by dendritic cells (DCs) to CD4+ Th cells occurs via the Major Histocompatibility Complex (MHC) class II complex and to CD8+ Tc cells via the MHC class I. T-cell activation requires not only presentation and recognition to the antigen presented by the antigen presenting cell (APC) but also activation of costimulatory molecules (CD80/CD28, CD40/CD40L) and cytokine recruitment (interleukin (IL)-1, IL-2, IL-6, IL-12, IFN-γ) [384, 386].

While activated CD8+ Tc cells (effector T cells) are able to recognize and lyse tumor cells by perforin and granzymes, activated CD4+ Th cells are able to modulate the antitumor response into a Th1 response or a Th2 response [387].

An antitumor response is promoted by a Th1 response and a pro-tumor response seems to correlate with a Th2 response. The CD3 complex serves as a T cell co-receptor which associates with the T cell receptor (TCR) and is useful as an identifier of T cells in general by CD3 antibody staining with IHC [388]. A CD45R0+ T cell refers to a subset of T cells that are memory T cells. Specifically, they are experienced T cells that have previously encountered an antigen and can upon a second encounter reproduce a fast and strong immune response.

It is still however debated whether the immune system eliminates continuously arising transformed cells. The timely elimination of pathogens and inflammation seems to be important so as to prevent the establishment of a constant inflammatory environment conducive to tumorigenesis [383, 384]. The tumor immunoediting concept explains why tumors still develop despite an active tumor immune surveillance in which the immune system specifically identifies and eliminates tumor cells on the basis of tumor-specific antigens or molecules induced by cellular stress. The three phases involved in tumor immunoediting, termed the 3 Es of tumor, are: elimination (cancer immune surveillance), equilibrium (cancer persistence) and escape (cancer progression) [389].

There is gaining evidence supporting the theory that cancer cells may escape the immune host responses by two mechanisms: immunoselection, selection of non-immunogenic tumor cell variants, and immunosubversion, active suppression of the immune response [328, 383]. Immunoselection refers to the induction of central or peripheral tolerance [390]. Central tolerance is when self-reactive T cells are deleted or converted to a regulatory phenotype in the thymus. Peripheral tolerance is seen when tumor growth can induce T cell tolerance in the periphery by mechanisms such as deletion, induction of antigen unresponsiveness, change of Tcell response.

Immunosubversion can be attributed to the tumor microenvironment such as expression of T-cell inhibitory molecules (such as B7-H1, HLA-G) by tumor cells, tumor antigen loss or downregulation of MHC molecules and suppressive factors such as transforming growth factor (TGF-β), VEGF, IL-10, indoleamine 2,3-doxygenase (IDO) expressed by tumor cells [383].

7.6.2 Immune response in CRC

Evidence for CRC immunoediting in humans is found in MSI tumors. Tumors with dysfunctional MMR genes (such as MLH1, MSH2) give rise to an accumulation of mutations that are not repaired in microsatellite repetitive DNA sequences thus resulting in microsatellite instability (MSI). As mentioned previously the MSI form of genetic instability is believed to be involved in the development of 10-15% of sporadic CRC. MSI tumors are often seen to be infiltrated by cytotoxic T lymphocytes (CD8+ T cells) and the presence of lymphoid follicles are common [232, 233].

Studies have shown that primary CRC patients with a dMMR status, or MSI status, have a better OS compared with patients with a pMMR tumor or MSS tumor [121, 246]. A theory is that the MSI defect causes high levels frameshift mutations potentially generating antigenic peptides thereby inducing the adaptive immune response (CD8+ T cells) which would then limit tumor growth and metastases [384].

An improved prognosis has been noted for patients with tumor infiltration by T cells, NK cells or NK T-cells for several tumor types [383]. Tumors with chronic inflammation and the presence of M2 macrophages seemed however to favor tumor growth and spreading [391]. The M2 macrophages secrete cytokines IL-4, IL-10, IL-13 and TGFβ and have an anti-inflammatory role as well as promoting angiogenesis. In contrast, M1 macrophages have a pro-inflammatory role, they secrete pro-inflammatory cytokines (IL-1, IL-6 and TNF) and are able to release reactive oxygen and reactive nitrate species.

The clinical importance of host response has been of interest especially in terms of tumor infiltrating immune cells. The presence of tumor infiltrating lymphocytes (TILS), where they can be found in the stroma, peritumoral and intratumoral areas, has been associated with decreased involvement of blood, lymph vessel and lymph node involvement [392].

The role of Treg cells is much debated in CRC as well as in other tumors due to conflicting reports. They are CD4+ and CD25+ T cells and express the nuclear transcription factor, Forkhead box P3 (FoxP3) by which they can be identified. The antigen specificity of TIL Treg cells is not yet established in humans. A theory is that the tumor microenvironment may influence them to be detrimental when blocking anti-tumor effector T cells or beneficial when decreasing chronic inflammation [384].

Poor prognosis in CRC has been associated with the presence of T_h17 cells [393].

7.6.3 Tumor infiltrating lymphocytes (TILS) in CRC

Several studies in cancer as summarized in a meta-analysis have demonstrated that a better prognosis is associated with the presence of peri- and intratumoral T cells (CD3+TH1 cells, CD8+cytotoxic T cells, CD45RO+ memory cells) [394].

In CRC all subsets of T cells are present both at the core and at the invasive margin of the tumor. The density of CD4+ memory T cells and CD8+ memory T cells (cytotoxic T cells) is decreased in T4-stage tumors (advanced local tumor invasion) compared with T1-stage tumors (limited local tumor invasion) [395]. Tumors seem to recur less if the primary tumor had high infiltrates (especially in the core) of CD4+ and CD8+ memory T cells [384, 394].

In stage II and III CRC an improved outcome is observed, less disease recurrence and a better OS, as the amount of intratumoral CD8+ T cells (cytotoxic) and memory T cells increased [396]. A better DFS and OS in high-risk stage II and stage III colon cancer was predicted by a higher quantity of lymphocytes in the tumor samples [397, 398].

CRC studies on stage II-III have shown a favorable outcome with higher CD3+ densities [399-403] or in both CD3+ and CD8+ [404] where four of these studies provided multivariate analyses which confirmed an independent prognostic value [399, 401-403].

One of these studies analyzed 587 patients stage I-II with CRC that had an MSS profile and found a high density of CD8+ to be an independent factor for better cancer specific survival (CCS) [403].

Two studies analyzing stage I-IV CRC showed a high density of CD8+ was correlated with an independent CCS [405] and OS [385]. One study analyzing CD3+ and CD8+ in stage I-IV, CRC was prognostic in univariate OS analyses but not in multivariate [406]. A study investigating in tissue microarrays (TMA) CRC stage II-III (n=445), Treg (FOXP3+) as well as CD8+ and CD45R0+ found that high density of Treg FOXP3+ in normal mucosa was associated with an independent adverse outcome while a high density in tumor tissue was associated with a better outcome [407]. They found high density of CD8+ and CD45R0+ to be prognostic only in univariate analyses, not in multivariate analyses. A similar larger study by Nosho et al studying CD3+, CD8+, CD45R0+ and Treg (FOXP3+) and using TMA, in stage CRC I-IV (n=768) found high densities of CD3+, CD8+, Treg (FOXP3+) to be prognostic of survival in univariate but not multivariate analyses while high densities of CD45R0+ were independently prognostic for survival [408].

In their multivariate analyses they adjusted for several molecular features such as KRAS, BRAF, PIK3CA mutations, MSI, CIMP and LINE-1 hypomethylation, all of which are associated with prognosis. They also found that MSI-H and LINE-1 methylation level are independent predictors of CD45R0+-cell density. Thus, the study supports the implication that a better survival due to a MSI-H profile could be due to tumor-infiltration of T-cells, specifically CD45R0+ T-cells.

Other studies in CRC, including stage I-II [409], II [410], III [411], and I-IV [409, 412] have also reported an improved survival with CD45R0+ cell density but have not adjusted to the molecular features named in the Nosho et al study [408]. One study evaluated DFS in patients (n=149) with colon cancer stage I-IV (I=43 patients, II=56 patients, III=34 patients, IV=16 patients) with regards to MMR-status and T-lymphocyte infiltration. They found a 5-year DFS of 59% for patients with pMMR or MSS tumors with no T-lymphocyte infiltration compared to DFS of 79% for patients with dMMR or MSI-H tumors with present T-lymphocyte infiltration [413]. The presence of a high lymphocyte density, measured using immune score, is indicative of a better prognosis in patients with CRC and seems more accurate than MMR-status itself [414].

7.6.4 Assessment of immune infiltrate

The Klintrup--Mäkinen (KM) grade has been used to assess the generalized inflammatory cell infiltrate at the tumor invasive margin in CRC. It has in studies shown prognostic value and is a semi-quantitative measurement done in H&E slides. It applies a 4-point scale ranging from low grade with no increase or mild increase in inflammatory cells to high grade with a prominent inflammatory reaction [415, 416].

Another method to assess T-lymphocyte infiltration, specifically CD3+ and CD8+ done by Ogino et al and Dahlin et al is also the method used in Paper IV [417, 418]. The method entails IHC of CD3+ and CD8+ T-cells that are then semi-quantitatively assessed under light microscope in representative areas at the invasive tumor front, tumor center and within the tumor epithelium. These 3 areas are scored according to score 1 as no or sporadic infiltration, score 2 as moderate infiltration, score 3 as abundant infiltration and score 4 as highly abundant infiltration. For each case the mean value of the added scores for

the subsites is then used to establish a total score for CD3+ and CD8+ T-cells. The total score with a range of 3 to 12, is then categorized as low expression (3-4), intermediate (5-6) or abundant (7-12).

The immunoscore (IS) method that also assesses densities of CD3+ and cytotoxic CD8+ Tcells by IHC has recently been internationally validated for colon cancer in a study done by Pagès et al [419]. The CD3+ and CD8+ T-cell densities are evaluated in the tumor and in the invasive margin but differs from the other methods in that quantification is done by digital pathology. A specially developed IS module integrated into an image-analysis system is used. The IS for each patient is calculated from the mean of four density percentile. The three-category IS analysis is scored with density of 0-25% as low, 25%-70% as intermediate and 70-100% as high while a two-category IS density of 0-25% is low and density of 25-100% as intermediate plus high.

The goal of the study was to establish IS as a reproducible, objective and robust method compared to manually assessing density of TILS which is subjective and less reproducible. The study in which 13 countries participated, analyzed IS in 2681 patients with stage I-III colon. The patients were divided into three sets, the validation set of 700 patients, the internal validation set of 636 patients and the external validation set of 1345 patients. A high level of reproducibility was observed between the observers and the centers. The finding in the training set of patients with a high IS having an independent significant lower risk of recurrence was validated in the two validation sets. It was independent to age, sex, T stage, N stage, MMR status and existing prognostic factors ($p<0.0001$). In stage II (n=1424 patients), the high IS patients had a significantly less 5-year risk of recurrence compared to the low IS patients (HR 0.33, 95% CI 0.21-0.52, $p<0.0001$; Cox multivariable $p<0.0001$).

Even a previous large CRC study with more than 4000 patients measuring density of CD3+ and CD8+ T cells using the method IS has validated the method and demonstrated that it was a stronger predictor of patient survival than MSI [414].

Thus, the IS is considered reliable and has possibly a higher contribution than the TNM-classification as an indicator for risk of recurrence of colon cancer. It strongly supports its implementation as a component of a TNM-Immune classification.

7.6.5 Immune checkpoint

Tumors have the ability to suppress an immune response by acquiring characteristics to escape detection or through activation of negative regulatory pathways that are called ‘checkpoints’ which are in turn key players in immune homeostasis [420, 421].

The treatment care of a number of malignancies which include melanoma, non-small-cell lung cancer, renal cell carcinoma has been revolutionized by immunotherapy. Positive results have been noted in dMMR/MSI-H mCRC when using immune checkpoint inhibitors, nivolumab a PD-1 blocking antibody or ipilimumab, a CTLA-4-blocking antibody.

Attention is especially focused on these two checkpoints, the cytotoxic T-lymphocyte protein 4 (CTLA4) and the programmed cell death protein 1 (PD-1). Activated T-cells express the cell-surface receptor PD-1 which can then bind to one of two ligands, PD-L1 and PD-L2 [422]. The PD-L1 ligand is expressed by tumor cells and immune cells after cytokine (e.g. interferon-γ) exposure. Expression of the PD-L2 ligand occurs for the most part on dendritic cells in normal tissue. An inhibitory signal is produced which impairs T cell activity. CTLA4 is a homolog of the co-stimulatory molecule CD28 which is only

expressed on activated CD4 and CD8+ T cells but is also constitutively expressed in Tregs. It competes with CD28 for binding to the shared ligands CD80 or B7.1 and CD86 or B7.2.

Anticancer immunity requires certain conditions to be effective. Three types of phenotypes have been identified by examining histological sections of tumor biopsies. They reflect specific underlying biological mechanisms. One phenotype is the 'immune-desert tumor' which is due to immunological ignorance, induction of tolerance or failure of appropriate T cell priming / activation. The immune-excluded tumor phenotype is caused by specific chemokine state, presence of barriers or vascular factors or stromal inhibition. The third phenotype is the 'inflamed tumor' in which infiltration of a variety of immune cells (including CD4 and CD8 T cells, myeloid cells, monocytic cells) and proinflammatory / effector cytokines are demonstrated. This scenario is believed to indicate an arrested pre-existing antitumor immune response that has been immunosuppressed in the tumor bed [422].

Response to anti-PD-1/PD-L1 therapy occurs mostly in patients with 'inflamed' tumors [423]. The presence of PD-L1 on infiltrating immune cells and at times tumor cells has been demonstrated [424]. A notable up-regulation of check point inhibitors such as PD-1, PD-L1, CTLA-4, LAG-3 and IDO has been demonstrated in the tumor microenvironment of MSI-H cancers [256].

In CRC the tumors with a dMMR/MSI-H status are often shown to be 'inflamed' with the infiltration of T-cells which accounts for a higher probability that they will respond to anti-PD1/PD-L1 or anti-CTLA4 therapy. Figure 10 illustrates mechanism of immune checkpoint inhibitor therapy [234].

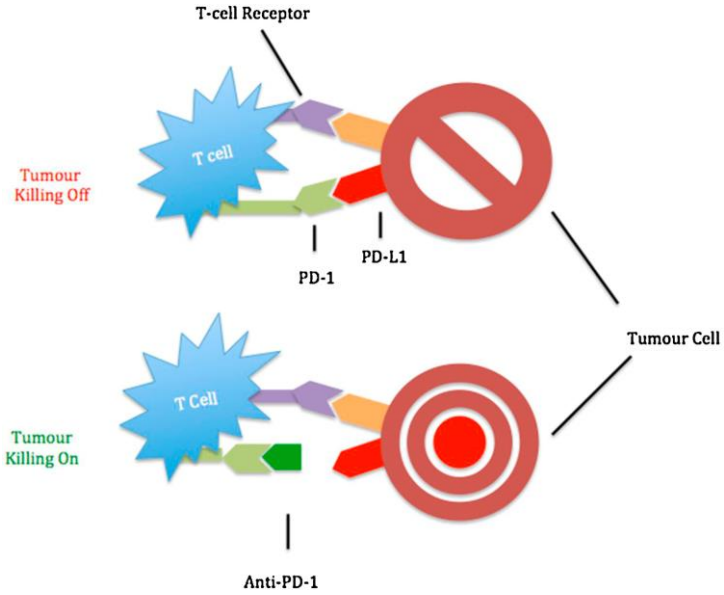


Figure 10. Mechanism of Immune Checkpoint Inhibitor Therapy. *(Reprinted, with permission, from Ryan et al, Crit Rev Oncol Hematol 2017)*

Before immunotherapy (top picture), the tumor cell's PD-1 ligand (PD-L1) molecule (red) binds to T-cells enabling the tumor cell to evade destruction by the immune system.
With immunotherapy (bottom picture), an anti-PD-1 inhibitor (green) blocks PD-L1 binding, thus enabling the T cell to target the tumor cell. Although, in this figure, the tumor cell is drawn as the source for the PD-L1, in some tumors such as dMMR/MSI-H CRC the main source may be macrophages or other TILs and myeloid cells).

The PD-L1 expression in MSI-H CRC has been found on TILS and/or myeloid cells, not on tumor cells [425].

8 AIMS OF THE THESIS

The overall aim of this thesis is to identify prognostic and predictive factors in primary CRC, stage II-III, so as to better define which patients, especially in stage II, would benefit from adjuvant chemotherapy.

Specific aims:

I. To analyze thymidylate synthase (TS) expression in primary CRC, stage II-III, as a prognostic / predictive factor of benefit for adjuvant chemotherapy.

II. To investigate if a combined rather than a single marker analysis of MMR-status and TS expression has a prognostic advantage and predicts response to 5-FU-based chemotherapy in stage II-III colon cancer.

III. To assess whether tumor budding grade in pMMR and dMMR primary CRC differed with regard to the development of local recurrence or metastases.

IV. To study the prognostic value of tumor budding, tumor border configuration and T-cell infiltration in stage II-III colon cancer with known MMR-status.

9 MATERIALS AND METHODS

9.1 Patients and Sample Material

Paper I to IV

The primary tumor samples analyzed for Paper I-IV were collected from patients who were included in the adjuvant CRC Nordic trials [426].

A total of 2224 patients with stage II-III CRC were randomized in the adjuvant Nordic trials to either surgery alone or surgery followed by 5-FU-based adjuvant chemotherapy during the period 1991-1997. Adjuvant chemotherapy was started within 11 weeks of surgery. Patients up to the age of 75 years were included.

Data censoring occurred at 120 months and follow-up ended November 2004.

The 1389 patients in Paper I, 716 patients in Paper II, 134 patients in Paper III and 478 patients in Paper IV are recruited from the original 2224 patients. See flow chart in Figure 11.

All of the studies performed for this thesis are retrospective.

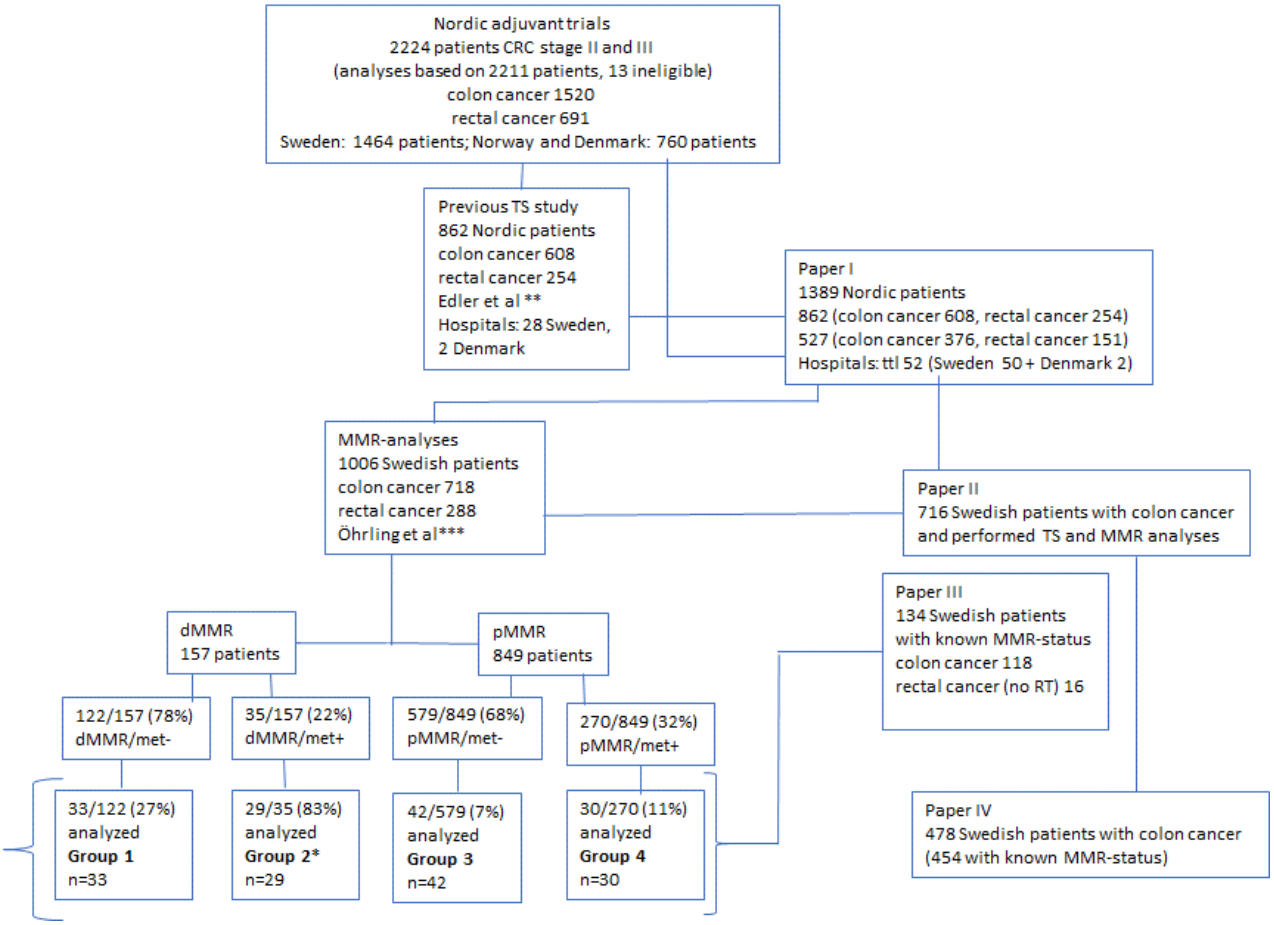
Tumor samples were retrieved from a total of 52 hospitals, 50 from Sweden och 2 from Denmark.

Parameters of clinical outcome were obtained from 6 Regional Cancer Centers in Sweden for studies in Paper I-IV and for studies in Paper I-II data for the included Danish patients was obtained from one Danish center of epidemiological oncology.

In Paper I, results of the analyses of 862 patients with CRC, stage II-III were previously reported by Edler et al [270]. Additional analyses of 527 specimens was performed for Paper I in order to achieve a larger group of patients (1389 patients) and a longer follow-up time.

In Paper II and IV only colon cancer Stage II-III was analyzed so as to avoid confounding factors such as pre-irradiated tumors.

Paper III was an exploratory study which was limited by the few available dMMR cases that developed recurrence or distant metastases. To increase available cases, rectal cancer was included, though only non-irradiated ones. The groups (see Figure 11) were matched according to age, gender and treatment arm.



Abbreviations: TS, thymidylate synthase; MMR, mismatch repair; dMMR, deficient MMR; pMMR, proficient MMR; RT, radiation therapy; met-, did not develop metastases or recurrence; met+, developed metastases or recurrence; Group 2*: index group identified and then matched to groups 1, 3 and 4 according to age, gender and treatment; **Edler et al [270]; ***Öhrling et al [236]

Figure 11. Patient Flow Chart for Paper I-IV.

For our studies (Paper I-IV) the tumor specimens obtained have been formalin-fixed, paraffin embedded tissue blocks.

Two sections, one section from each of the two available paraffin-embedded tissue blocks), each 4 µm-thick, were taken from different parts of the tumor for the TS and MMR IHC analysis.

For the tumor budding analyses in Paper III and Paper IV as well as for the tumor border configuration and T-cell infiltration, two paraffin blocks representing the tumor invasive regions were available for the majority of the cases. The respective sections which were stained with H&E were reviewed by investigators (DE MH) for Paper III and by investigator/pathologist (AM) for Paper IV. One section/block per case with the maximum tumor invasion was then chosen before performing immunohistochemical analysis.

The number of tumor material obtained for TS analyses and MMR analyses was dependent on what was submitted by the treating institutions that participated in the Nordic trials. For studies in Paper III-IV less tumor material was available as some paraffin tumor blocks had been returned to the original treating institutions.

For Paper I-II the 6th edition of the AJCC staging system was used. The 7th was used for Paper III-IV.

A pathologist (AM) histologically reviewed the cases for Paper IV and contributed to the addition of mucinous status as well as T-stage.

Patient and tumor characteristics for Paper I-IV are summarized in Table 7.

The studies, Paper I-IV were approved by the ethics committee at Karolinska Institutet, Stockholm.

Table 7. Patient and tumor characteristics Paper I-IV.

Characteristics		**Paper I**	**Paper II**	**Paper III**	**Paper IV**
Total		1389	716	134	478
Age:	Median	65	66	66	66
	Below median	635 (46%)	354 (49%)	67 (50%)	229 (48%)
Gender:	Male	782 (56%)	379 (53%)	69 (51%)	257 (54%)
	Female	607 (44%)	337 (47%)	65 (49%)	221 (46%)
Tumor site:	Colon	984 (71%)			
	Proximal Colon		389 (54%)	71 (53%)	266 (56%)
	Distal Colon		327 (46%)		212 (44%)
	Distal Colon + Rectum			63 (47%)	
	Rectum	405 (29%)			
Stage:	II	678 (49%)	356 (49%)	43 (32%)	225 (47%)
	III	711 (51%)	360 (50%)	91 (68%)	253 (53%)
No. analyzed LN:	<12	896 (88%)	459 (86%)	84 (80%)	293 (85%)
	≥12	123 (12%)	72 (14%)	21 (20%)	53 (15%)
	Unknown	370	185	29	132
Tumor grade:	G1 (well diff)	87 (6%)	57 (8%)	6 (5%)	44 (10%)
	G2 (moderately diff)	958 (73%)	489 (71%)	90 (68%)	323 (71%)
	G3 (poorly diff)	274 (21%)	144 (21%)	36 (27%)	88 (19%)
	Unknown	70	26	2	23
Treatment:	Surgery alone"	708 (51%)	370 (52%)	62 (46%)	255 (53%)
	Plus adjuvant CT	681 (49%)	346 (48%)	72 (54%)	223 (47%)
TS Grade:	0-1 (low)	399 (29%)			
	2-3 (high)	990 (71%)			
	0-2 (low)	929 (67%)	443 (62%)		
	3 (high)	460 (33%)	273 (38%)		
MMR-status:	dMMR		142 (20%)	62 (46%)	90 (20%)
	pMMR		574 (80%)	72 (54%)	364 (80%)
	Missing data				24
Tumor budding:	<5 (low)			*64 (48%)	
	≥5 (high)			*70 (52%)	
	<10 (low)			*101 (75%)	**346 (72%)
	≥10 (high)			*33 (25%)	**132 (28%)
Tumor border configuration:					
	Infiltrating				192 (40%)
	Pushing + mixed				286 (60%)
Total CD3 Score:	3-4 (low)				113 (24%)
	5-6 (intermediate)				237 (49%)
	7-12 (high)				128 (27%)
Total CD8 Score:	3-4 (low)				148 (31%)
	5-6 (intermediate)				222 (46%)
	7-12 (high)				108 (23%)

Abbreviations: CT, chemotherapy; diff, differentiation; LN, lymph nodes; MMR, mismatch repair; *tumor budding grading in Paper III; **tumor budding grading in Paper IV

9.2 Method Immunohistochemistry (IHC)

A common method to detect specific proteins in histological intact tissue is the method of IHC. It applies the concept that antibodies, immunoglobulins created by the immune system, can be produced that bind to these specific proteins and are then visualized by a reagent attached to the antibody.

It is a technique that has been used since the 1940s and is routinely used as a critical tool in health care and pathology for diagnostic purposes as well as for optimizing a patient's treatment. Although visualization of targets in tissues using IHC is an established method, it is important that new assays require optimization depending on the tissue analyzed as well as the target protein [427].

It is the chromogenic stain that can be observed in light microscopy and be used to semi-quantitatively determine protein expression and has the stability allowing it to be reviewed years later.

The advantage of IHC on tissue slides compared to the method of tissue microarray (TMA) which uses limited tumor tissue, is that it allows for a visual presentation of the target as well as the expression pattern among heterogeneous cell populations.

Choice of antibody to detect the correct molecule of interest with good specificity and affinity is of importance in order to have a reliable interpretation of the assay. Optimization and titration of the antibody concentration is needed. If too much antibody is added it will increase the possibility of low-affinity off-target binding occurrences while too little antibody can lead to a false negative result.

The two main types of antibodies used are the monoclonal or the polyclonal. Monoclonal antibodies bind to the same epitope while polyclonal antibodies bind to different epitopes on the target. The advantages of using polyclonal antibodies are that they are often very potent and can outweigh potential background noise but the disadvantage is their limited resource as they are derived from animal sera. Monoclonal antibodies are produced in hydridoma cell lines providing the advantage of continuity of production and are often well defined as to their epitope binding site. One still though has to be aware in cases of low specificity or if the target epitope is present in low amounts.

The method of IHC was used in Paper I-IV

The procedure entails that tissues upon retrieval from the patient are formalin fixed in paraffin-embedded tissue blocks so as to preserve epitopes and morphology as well as to prevent degradation. The paraffin blocks are then sectioned using a microtome into thin slices, about 4-10µm. **In our studies 4µm slices were used**. The slides are then mounted on glass slides, dried at room temperature and then baked for about an hour at 60ºC.

Retrieval of the desired antigen or epitope requires first demasking of the epitopes so as to allow the primary antibody to bind its target. The first step is deparaffination of the tumor sections using xylene followed by rehydration in ethanol and washing in distilled water. **This was done for all of the tumor sections included in Paper I-IV.**

The tumor slides (for Paper I-IV: TS MMR and MNF-116) were then incubated in 3% hydrogen peroxide to inhibit endogenous peroxidase activity.

Retrieval of the antigen is carried out in citrate or EDTA buffer for 20 minutes in a microwave oven followed by 20 minutes of cooling at room temperature. This part of the process breaks down the molecular cross links formed by formalin fixation.

For Paper I-II, regarding the TS expression analysis and in Paper III-IV with MNF-116 staining, citrate buffer (pH 6.0) was used for antigen retrieval while for Paper II-IV, regarding MMR expression analysis, 1mM EDTA (pH 9) was used.

Thereafter an overnight incubation (e.g. at 4ºC for TS and MMR analysis, **Paper I + Paper I-IV**) is done with the specific primary antibody. See section 9.2.1.

Before incubation with the primary antibody in the TS analysis (**Paper I-II**), the slides were then blocked with 1.5% horse serum (same species as secondary antibodies) to reduce nonspecific background staining.

After incubation with the primary antibody, the samples are rinsed and then incubated with the secondary antibody.

This entailed for:

- TS (**Paper I-II**): incubation with biotinylated horse antimouse secondary antibodies for 30 min, at room temperature.
- MMR (**Paper II-IV**): incubation for 30 min at room temperature with an amplification system with a labelled polymer, EnVision™*/HRP rabbit/mouse and goat/rabbit, (DakoCytomation, Denmark).
- MNF-116 (**Paper III-IV**): incubation for 30 min at room temperature with the amplification system (EnVision + System-HRP Dako A/S, EnVision™*, Dako, CA, USA).

*EnVision system: a two step procedure where after primary antibody is added the polymeric conjugate in EnVision consists of peroxidase molecules and secondary antibody molecules bound directly to an activated dextran backbone. The stepwise procedure is meant to eliminate the problem of endogenous biotin [428].

Upon binding of the biotinylated secondary antibody to the primary antibody, the reaction of peroxidase with biotin causes visible staining

The next step done in IHC analyses for Paper I-IV was to visualize the sites of bound peroxidase. Therefore, slides were washed and immersed in 0.05% 3,3'diaminobenzidine tetrahydrochloride (DAB), a chromogen, which is converted into a brown / bluish precipitate at the site of the reaction. The last step is counterstaining with hematoxylin which stains cellular cytoplasm a pale bluish color and stains cell nuclei a darker blue thus facilitating observation of histological features. See Figure 12 for an illustration of IHC.

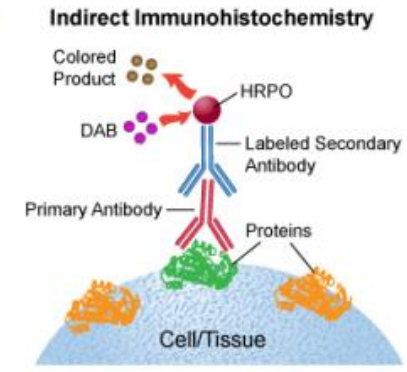


Figure 12. Indirect IHC.

In **Paper IV** the IHC procedures for staining CD3+ and CD8+ T cells involved a staining machine (Ventana BenchMark Ultra, Ventana Medical Systems, Inc., Tucson, AZ, USA) utilized with the CC1 standard pretreatment and the iVIEW DAB detection kit (Ventana Medical Systems, Inc.) for visualization.

9.2.1 Primary antibody in PAPER I-IV

9.2.1.1 TS

The primary antibody we used to determine TS protein expression in Paper I-II as well as the previous TS study by Edler et al [270] was a mouse monoclonal antibody TS106 that detects TS of human origin (produced by NeoMarkers, Fremont, CA, USA), used at a dilution of 1:75.

Data did not show any disadvantage between using the monoclonal TS antibody versus the also available polyclonal rabbit-antihuman TS antibody [429].

9.2.1.2 MMR

The primary antibody used was a mouse immunoglobulin G monoclonal antibody:
MLH1 (clone G 168-15; used at dilution 1:100), supplied by PharMingen, ,San Diego, CA, USA

PMS2 (clone A 16-4; used at dilution 1:75), supplied by PharMingen, San Diego, CA, USA

MSH2 antibody (clone FE-11; used at dilution of 1:100), supplied by Oncogene, Research Products, Boston, MA, USA.

9.2.1.3 Tumor budding

For Paper III and IV IHC was performed to aid in the detection of tumor budding by staining pan-cytokeratin with monoclonal mouse antibody MNF-116, used at dilution 1:75, produced by DakoCytomation, Glostrup, Denmark.

9.2.1.4 T-cell, CD3 and CD8

In Paper IV IHC was done to detect CD3+ T cells and CD8+ T cells with the primary polyclonal CD3 antibody (used at dilution of 1:50), produced by Dako, Glostrup, Denmark) and the primary polyclonal CD8 antibody (Clone C8/144B, used at dilution 1:50), produced by Dako, Stockholm, Sweden.

9.2.2 Other methods to measure TS, MMR

The testing methods to determine a defect in MMR include besides IHC, PCR (touched upon in section 2.3.1) and next-generation sequencing. Which method is used is determined by the institutional availability.

Next generation sequencing is becoming more available at academic institutions, hospitals and even for commercial use. It entails more of a broad genomic sequencing that detects MSI as well as many other mutations. The importance of genetic testing and counseling (e.g. Lynch syndrome) has to be taken into account as testing becomes more prevalent.

A method that has been used in several studies to analyze TS mRNA expressions is the semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). It is a valid and reliable method and has the advantage that it requires small amount of tissue to do the analysis [430]. The TS mRNA sequence serves as a template for reverse transcriptase generating a single-stranded DNA (cDNA) which in turn serves as a template for PCR. To identify the mRNA of interest, a primer is used with known coding regions. PCR is widely used as the method to exponentially amplify the DNA sequence of interest. The PCR process relies on thermal cycling that involves a DNA primer that is complementary to the

target DNA strand, a heat-stable Taq DNA polymerase and free nucleotides. Classical post-PCR steps involve electrophoretic separation and precise quantification of electrophoretic bands. Another method that is widely used is high-performance liquid chromatography (HPLC)-based procedures which separates and quantifies PCR products by liquid chromatography.

The quantitative gene expression is normalized to the expression of 'housekeeping genes' such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

9.3 Scoring

9.3.1 TS (PAPER I-II)

The method developed by Johnston et al [281] was used for visual, performed under light microscope, grading of the staining intensity of TS protein expression with a scale from 0 to 3 (grade 0 = no staining, grade 1 = weak, grade 2=moderate, grade 3=intense staining). This method was applied in the previous study by Edler et al [270] with 862 specimens as well as for the 527 specimens analyzed in Paper I. Taking into account TS expression heterogeneity [431], analysis was done in two different areas from each primary tumor. A previous study by our group found an increase from 81% to 96% for detecting a maximal area of TS staining with a single tumor sample respective two tumor samples [431].

A granular cytoplasmic staining pattern was observed with the monoclonal antibody TS106. Normal colon epithelium shows a weak staining or no staining while TS staining can be observed in lymphocytes and macrophages 'adjacent' to the tumor.

The highest intensity found in the tumor established the grade of the tumor even if the area of high intensity was small. Scoring was done by four of the authors (MK, KÖ, DE, MH) who were blinded to clinical and pathological data.

Each time a set of tumor samples was stained two reference slides were included with previously determined staining intensity of TS grade 0-1 and TS grade 2-3 as controls.

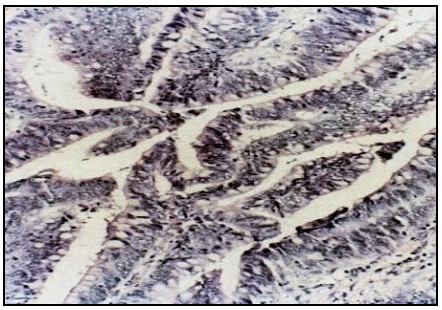
The observers had a ≥90% level of agreement in scoring. Resolving of scoring discrepancies was done by consensus after re-examination.

The cut-off of low grade versus high grade TS intensity has been TS 0-1 versus TS 2-3, respectively in the majority of studies [269, 276] and was applied in Paper I.

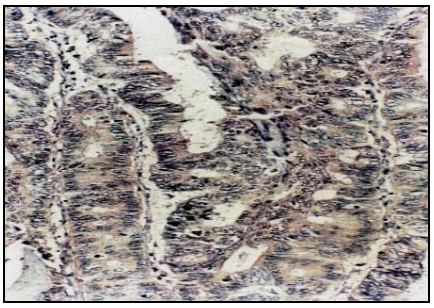
The categorization of TS grade 0-2 (low grade) versus TS grade 3 (high grade) as used in the study by Allegra et al [276] was used in Paper II and was an additional categorization used in Paper I.

An observation noted while TS grading was performed is that grade 0 and grade 3 were relatively uncomplicated to discern while grade 1-2 were more difficult.

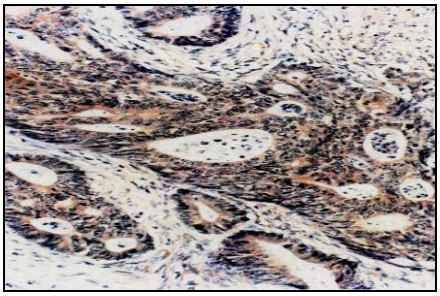
Figure 13 illustrates TS grades 0-3.



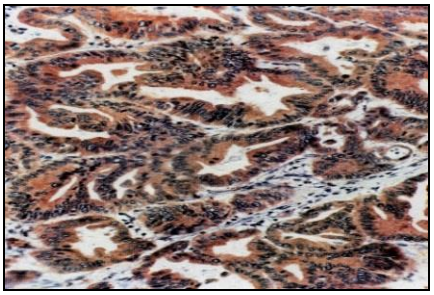
TS grade 0



TS grade 1



TS grade 2



TS grade 3

Figure 13. TS grading 0-3.

9.3.2 MMR (PAPER II-IV)

Two different areas from each primary tumor were analyzed for MMR protein expression considering the described intratumoral heterogeneity of its expression [432].

Examination was done under a light microscope and scored by two independent observers (KÖ, MH, DE) who were blinded to clinical and pathological data. A deficient MMR expression was defined by the tumor cells displaying a complete absence of nuclear staining with the respective monoclonal antibody. The internal positive control used was the intact nuclear staining of normal tissue (including nonneoplastic stromal cells and lymphocytes) adjacent to the tumor. A staining was considered positive if any area of the tumor displayed positive staining.

Figure 14 displays the presence versus the absence of nuclear staining for the MMR-protein, MLH.

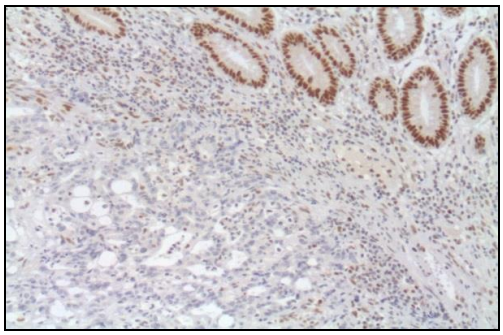
For Paper II-IV, the IHC method was used to detect MMR protein expression of MLH1 and MSH2 and when confirmation was needed of MLH1 deficiency even some cases of PMS2 were analyzed due to the known association between the two proteins (see section 2.3 for more information on the interaction of the different MMR-proteins).

Data supports that the majority of dMMR cases are detected upon staining the two proteins, MSH2 and MLH1, with a reported sensitivity of 92% and a specificity of 100% [433].

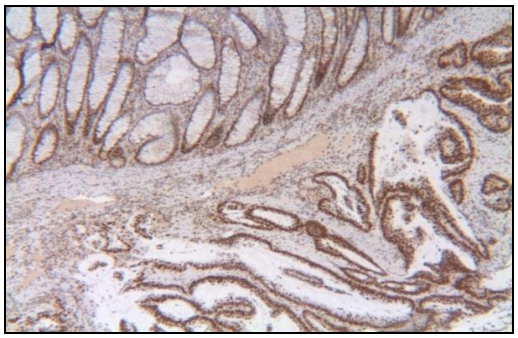
Assessment of the nuclear staining of MSH2 was uncomplicated while MLH1 staining was more difficult to interpret as also reported in other studies [434]. For the 51 cases with unclear MLH1 status, staining of PMS2 was performed considering the functional interaction between MLH1 and PMS2 as described earlier. MLH1 staining was considered negative if PMS2 was found negative. One should note that it is unclear whether the IHC analysis of PMS2 gives the best clarification since, in sporadic CRC, promoter methylation

is responsible for the loss of protein expression of MLH1. In routine screening for Lynch syndrome all four MMR proteins (MSH2, MLH1, PMS2, MSH6) are analyzed in which mutations in PMS2 are relatively infrequent.

For Paper IV MMR status was available for 454 (95%) of the 478 colon cancer cases.



MLH1 negative



MLH1 positive

Figure 14. Illustration of IHC staining for MLH1.

9.3.3 Tumor budding (PAPER III-IV)

Studies done on tumor budding in CRC have used several different methods for assessing it as well as different cut-off values for defining high-grade versus low-grade tumor budding. The methods either had a qualitative approach or a quantitative approach.

For both Paper III and IV we chose as other studies have also done [329, 335, 435, 436] to highlight the neoplastic epithelium by pan-cytokeratin staining in order to better identify tumor buds and avoid their underestimation.

Consultation was done with a pathologist before Paper III to establish a suitable method for quantitative assessment of tumor budding.

More consensus on methodology in the studies of tumor budding in CRC was observed in preparation of Paper IV, in which guidelines published by Karamitopoulou et al [340] gained interest and were applied in Paper IV.

The International Tumor Budding Consensus Conference (ITBCC) has recently published guidelines for assessment and scoring of tumor budding so as to standardize the method and aid in its clinical implementation [341]. It uses H&E slides, analyzes tumor budding in one hotspot field with a specific size of 0.785mm^2 and categorizes low-grade budding as 0-4 buds, intermediate-grade as 5-9 buds and high-grade budding as ≥10 buds.

Examples of slides depicting low-grade budding and high-grade budding are shown in Figure 15.

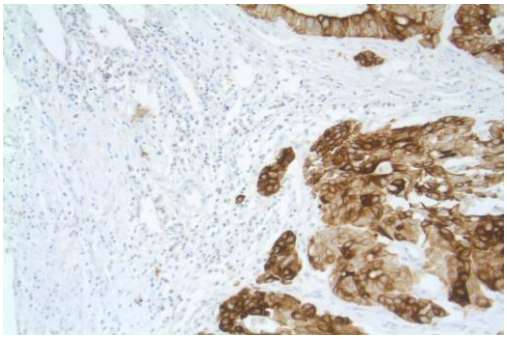


Fig A. Low-grade tumor budding, 20x

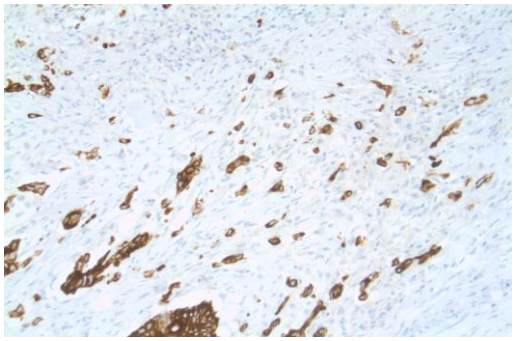


Fig B. High-grade tumor budding, 20x

Figure 15. A. Illustrates low-grade tumor budding. **B**. High-grade tumor budding.

9.3.3.1 PAPER III

Two independent investigators (MK and CL) blinded to the clinical and pathological data performed the assessment of tumor budding with MNF-116 staining. Isolated single de-differentiated cancer cells or cluster of fewer than five such cells at the invasive front were defined as tumor budding [309].

Scanning of the invasive front of the two available tumor samples per case was performed with a multihead microscope at low power magnification (10x) to identify the most suitable sample (containing maximum tumor invasive front). The chosen tumor sample was then used for counting the number of tumor buds. Counting of the tumor buds, at magnification of 20x, was done in a specified area (0.05mm^2) defined by a grid along the entire invasion margin of the tumor. The median tumor budding value of 5 found in this study was used as a cut-off as well as the cut-off of 10, an optimal cut-off considered in other studies using H&E stained slides [309, 367] and cytokeratin staining [340].

9.3.3.2 PAPER IV

Two paraffin blocks per case with representative colorectal tumor were available for most of the cases. The respective sections which were stained with H&E were reviewed by pathologist AM and one section/block per case with maximum tumor invasion was chosen. Thereafter, the 4um sections were cut and mounted on slides from the selected blocks and IHC staining with MNF-116 was performed as previously described.

For Paper IV it was possible to apply digital imaging technology which allowed the investigators to share and study the specimen with improved accuracy.

Digital pathology where images can be uploaded with appropriate software application is becoming more common in clinical use. It has the advantage of eliminating degradation of samples, it preserves quality and the images can be shared when needed to improve diagnostics and as an education tool.

Slide digitalization in Paper IV of the MNF-116 stained slides was done with a Vslide slide scanning microscope (Metasystems, Alltlussheim, Germany) which scanned slides with a x10 objective and RGB led illumination for color deconvolution. Metaviewer software (Metasystems, Alltlussheim, Germany) converted the scanned digital slides into images of .tif format at a resolution of 0.65 micrometers per pixel.

To assess tumor budding for Paper IV the guidelines published by Karamitopoulou et al were applied [340]. This entailed identifying the average number of tumor buds in 10-high-power-fields (HPF) of view with most tumor budding (hotspots) along the invasion front.

In Paper IV tumor budding was defined as a single detached cancer cell or a cluster of ≤5 detached tumor cells [311].

HPF commonly denotes to x40 objective of the conventional microscope. The area of the field of view at x40 of the conventional microscopes used at the local pathology departments, was computed to be 0.49mm^2 and was then used to simulate region of interest on the digital slide. The process involved thereafter was as follows:

- Use of Photo Shop to view the .tif images at low magnification to localize the invasive margin of the tumor.
- Identification of higher tumor budding regions at low/medium magnification.
- Use of Photo Shop to digitally define, using a colored circle, the 10-HPF regions where each HPF had a defined area of 0.49mm^2.

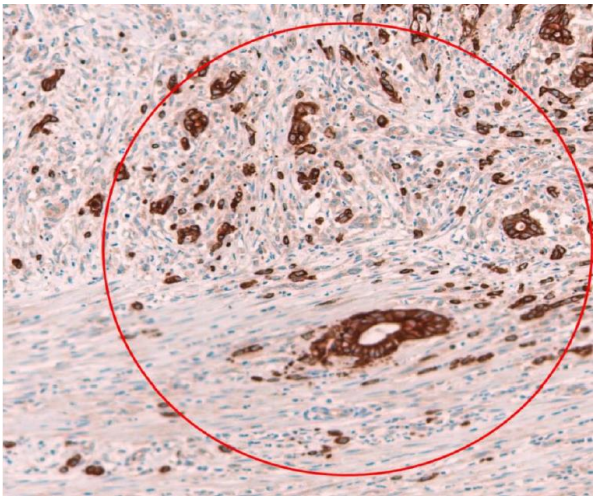
The digital images were reviewed by two independent investigators (MK and AM) blinded to the clinical and pathological data. For each field of view the number of tumor buds was recorded and the average number of buds in the 10-HPF per case was used to determine grade of tumor budding. As in Karamitopolou et al ([340]) high grade tumor budding was defined as ≥10 buds. In the few cases of discrepancy, scoring was resolved by consensus after re-examination.

Figure 16 illustrates low-grade and high-grade tumor budding in 10 HPF as well as high-grade tumor budding in one HPF.

A substantial agreement on budding count was observed between the investigators p=0.006, r=0.99, ICC=0.99.

A. 10 HPF, low-grade tumor budding, High power field (HPF).

B. 10 HPF, high-grade tumor budding. High power field (HPF).



C. 1 HPF, high-grade tumor budding. High power field (HPF).

Figure 16 A-C. Tumor budding examples in PAPER IV.

(A) 10 HPF, low-grade tumor budding, High power field (HPF).
(B) 10 HPF, high-grade tumor budding. High power field (HPF).
(C) 1 HPF, high-grade tumor budding. High power field (HPF).

9.3.4 Tumor border configuration (PAPER IV)

The same digital slides used for tumor budding in Paper IV were used to assess tumor border configuration by two independent investigators (MK and AM) blinded to the clinical and pathological data.

Application of the method described by Morikawa et al [375] was used for assessment. This entailed evaluation of the invasive tumor border at low magnification (x10) and choosing the category which best represents the growth pattern: pushing (expansive), intermediate or infiltrative.

The growth patterns are identified by the following features:

- pushing: tumors with well circumscribed growth
- infiltrative: irregular cords and clusters of cancer cells and small glands without a distinct border
- intermediate: presence of the irregularity of the large and medium-sized glands at the invasive border

9.3.5 T-cell infiltration (PAPER IV)

Semi-quantitative analysis of the IHC stained CD3+ and CD8+ T-cells was done under light microscope by an independent observer (AL) blinded to clinical and pathological data. The most representative areas were chosen from 3 subsites:

- the **invasive tumor front**: identified as the stromal area along the invasive margin defined by a depth of two HPF (x40 objective) underneath the invasive margin
- the **tumor center**: defined as the stromal area within the tumor mass between, as well as clearly separated from the luminal border and the invasive front
- **within the tumor epithelium**: defined as intraepithelial lymphocytes located within tumor cell nests

Scoring of T-cell infiltration was assessed according to Dahlin et al [418] as follows:

- score 1: no or sporadic infiltration
- score 2: moderate infiltration
- score 3: abundant infiltration
- score 4: highly abundant infiltration

All sections were examined twice (AL) and in cases of discrepant scoring, cases were re-evaluated before setting a conclusive score.

As described in Dahlin et al and Ogino et al [417, 418], the mean value of the added scores for CD3+ and CD8+ T-cells for each subsite (tumor front, center, intraepithelial) was calculated to reach a total CD3+ score and a total CD8+ score. The total score ranged from 3 to 12, in which low expression was defined as 3-4, intermediate as 5-6 and abundant as 7-12. Each case depending on its score was then placed into the appropriate category.

9.4 Statistical Analysis

Statistical analyses were done for all papers included in this thesis. Statisticians were consulted (Bo Nilsson for Paper I-II-III, Yunxia Lu for Paper III and Hemming Johansson for Paper IV) concerning the suitable choice of statistical analysis.
All statistical tests were performed using Statistica (StatSoft Scandinavia AB, Uppsala, Sweden), version 7 (Paper I and II) and version 10 (Paper III and IV).

Paper I-IV

The Chi-square test was used to compare the differences in distributions in the different groups which are considered unordered categorical variables.
Statistical tests were two-sided in which differences with values of $p<0.05$ were considered statistically significant.

DFS was defined as time from surgery to the first event of local recurrence, presence of distant metastases or death of any reason and OS was defined as time from surgery to death.

The Gehan-Wilcoxon univariate test was used:

- in Paper I to analyze relationships between survival and patients demographics and tumor characteristics and
- in Paper II to examine the possible correlation between OS and the combined analysis of MMR status and TS expression.

Cox univariate regression analysis was used in Paper III and IV to examine the relationships between OS and DFS and patients' demographics and tumor characteristics.

Survival curves were constructed with the Kaplan-Meier method. The logrank test was used to compare the difference between groups. Both the Kaplan-Meier method and the logrank test are examples of univariate analysis, ignoring the impact of other factors.

Multivariable survival analyses were performed using the Cox proportional hazards regression model for calculation of hazard ratios (HR). A method which allows several variables to be evaluated simultaneously as to how they influence the rate (HR) of an event (e.g. death) at a specific point in time.

Paper II

To analyze the relationship between MMR status and TS expression we used the Spearman's rank test."

Paper III-IV

Linear correlation between the two observers' tumor budding counts was assessed using Pearson correlation coefficient (r). The intra-class correlation coefficients (ICC) assessed interobserver agreement, in which values range from 0 (no agreement) to 1 (perfect agreement).

10 RESULTS AND DISCUSSION

10.1 PAPER I

Prognostic and Predictive value of Thymidylate Synthase Expression in Primary Colorectal Cancer

Objective: To investigate thymidylate synthase (TS) expression in primary CRC as a prognostic and predictive marker of benefit for adjuvant chemotherapy in a large group of patients with a longer follow-up time.

Results: Analyses of 862 of the patients in this study have been previously reported [270]. In this study including 1389 patients (862 patients with the addition of 527 patients), 616 patients died of whom 497 (81%) of them died of CRC during the median follow-up time of 75 months (range 1-120). The majority, 984 (71%), of the tumors were located in the colon. Of the 405 patients with rectal cancer, 214 (53%) were treated with preoperative radiation (5Gyx5).

A better OS was independently significantly linked to the female gender, younger age (<65 years), well differentiated tumors (low grade), stage II versus stage III and the removal of ≥12 lymph nodes.

In the entire study population, TS expression grade 0-1, defined as low, was found in 399 (29%) primary tumors while TS expression grade 2-3, defined as high, was found in 990 (71%) primary tumors. With the classification of TS expression grade 0-2 as low, we found 929 (67%) tumors and with TS expression grade 3 as high we found 460 (33%) tumors. The majority, 530 (38%) of tumors had a TS expression of grade 2 while 21 (2%) had TS expression grade 0 and 378 (27%) had TS expression grade 1.

TS expression was found to be independent of tumor localization and stage and in rectal cancer patients, it did not differ regardless of whether they received or not received preoperative RT. No method of TS classification found TS expression to be prognostic for OS or DFS in the entire study group.

Analysis of the prognostic value of TS expression in the patients treated with surgery alone (n=708) and in those treated with surgery followed by adjuvant chemotherapy (n=681), showed low TS expression (0-1 or 0-2) to be independently associated with a longer OS and DFS, only for the patients in the surgery alone arm (p=0.045 or p=0.002, respectively) (Figure 17 A and B). No difference in OS was found in adjuvant chemotherapy patients regardless of whether the patients received ≥90% of the planned FU-dose (n=335) versus <90% (n=288) of the dose (n=58, excluded patients due to insufficient FU-dose information).

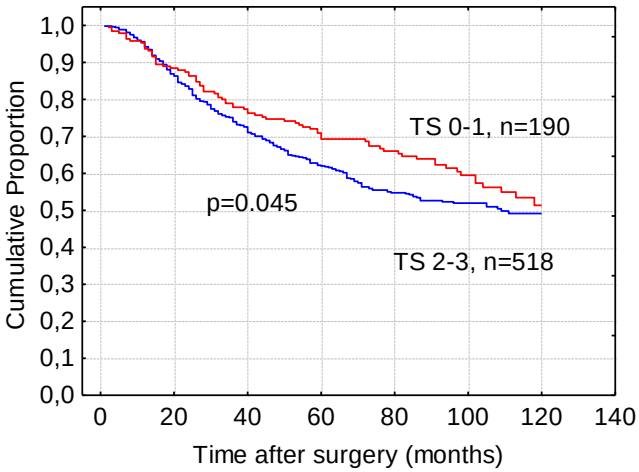


Figure 17. (A) Overall survival (OS) in 708 patients with colorectal cancer stage II and III treated with surgery alone according to expression of TS 01-2 versus 2-3.

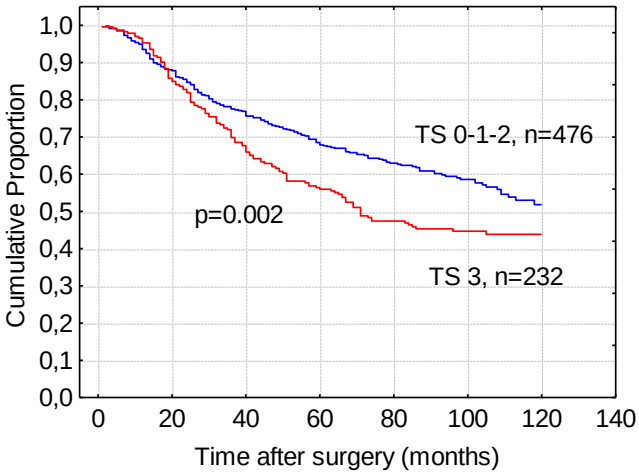


Figure 17. (B) Overall survival (OS) in 708 patients treated with surgery alone according to expression of TS 0-1-2 versus TS 3.

Comparing the surgery alone group to the adjuvant chemotherapy in the low TS expression (grade 0-1 or grade 0-2), no difference in OS or DFS was found. However, this comparison in the high TS expression (grade 3) group showed a significant longer OS for the adjuvant chemotherapy group compared to the surgery alone group (p=0.0005) (Figure 18 B). It remained significant in the multivariate analysis (p=0.0008). The same analysis remained independently prognostic in the subgroups of colon cancer stage II-III (p=0.007), colon cancer stage III (p=0.005) and rectal cancer stage II (p=0.01), but not in colon cancer stage II, rectal cancer stage II + III and rectal cancer stage III.

Analysis in the high TS grade group (grade 2-3), showed only a tendency to a longer OS for the group treated with postoperative adjuvant chemotherapy compared to surgery alone (p=0.07) (Figure 18 A).

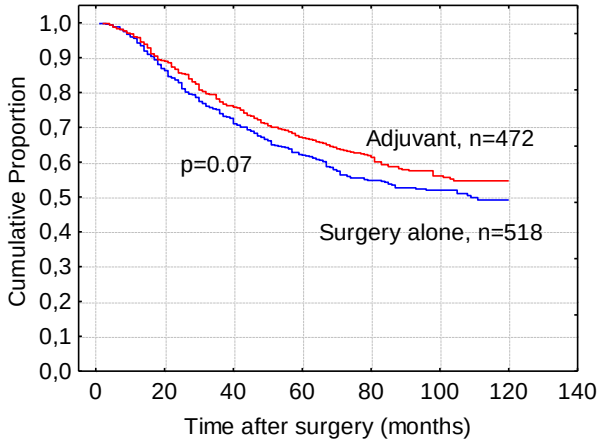


Figure 18. (A) Overall survival (OS) in the group of 990 patients with high TS expression (TS 2-3) tumors according to treatment with surgery alone versus surgery plus adjuvant chemotherapy.

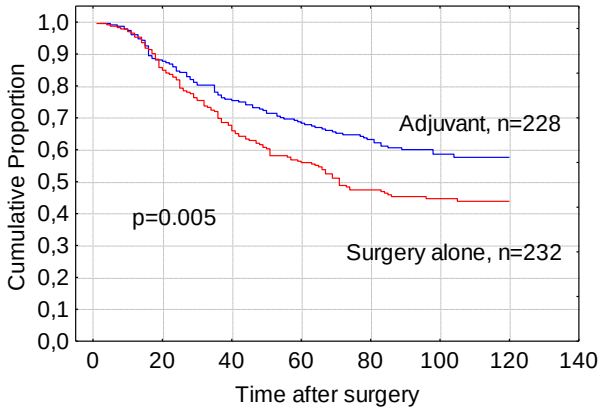


Figure 18. (B) Overall survival (OS) in the group of 460 patients with TS grade 3 tumours and treatment with surgery alone versus surgery plus adjuvant chemotherapy.

Discussion: Our finding that TS was not prognostic in our enlarged study population of 1389 primary CRC patients or in the group treated with surgery followed by adjuvant chemotherapy was in contrast to our previous study of 862 patients in which a low TS expression (grade 0-1) correlated with a longer OS (p=0.04) [270].

Popat et al's meta-analysis from 2004 which included both CRC adjuvant and metastatic disease settings with a total of 3497 patients, concluded that patients with high levels of TS expression had poorer OS compared to ones with low levels [269]. One study included in the meta-analysis, using IHC to analyze TS expression in 465 colon cancer patients who were randomized to surgery alone or plus adjuvant chemotherapy also failed, as in our study, to show a significant association between TS expression and outcome [274]. Furthermore, another large study published after Popats meta-analysis including 779 patients with TS analyzed by IHC using a polyclonal antibody found no association between TS expression and survival [268]. However, a colon cancer adjuvant study included in Popats meta-analyses using the IHC method in tumor material from 706 randomized patients, found high TS grade (grade 3) to be prognostic of a shorter OS [276].

Isolating the adjuvant cases (2610 patients) in the meta-analysis, Popat et al found, as in our study, that high TS expression predicted worse OS for patients treated with surgery alone but not for patients treated with surgery followed by adjuvant chemotherapy [269].

In contrast to our finding that low TS expression (using both grade 0-1 and grade 0-2 as low) correlated to a better OS and DFS for the surgery alone group of patients, another large study found low TS expression (grade 0-2) correlated with a worse prognosis for this group [290]. Their study used TMA as tumor material to analyze TS by IHC from 945 CRC patients, who were non-randomly, treated with or without FU-based chemotherapy. An

explanation for their differing result could be that there is a risk of underestimating the true frequency of high TS expression using TMA since it analyzes a limited area of tumor tissue thus not accounting for heterogeneity and focal staining.

There are conflicting results in studies as to whether TS expression in the primary tumor can predict response to FU-based regimens in the adjuvant setting [269]. In our study we found the trend of benefit of adjuvant 5-FU-based chemotherapy to be greater than in our previous smaller study for patients whose tumors expressed TS grade 2-3. With the classification of high TS as grade 3, we found a significant survival for patients receiving adjuvant chemotherapy compared to surgery only. Several studies support a survival benefit with adjuvant 5-FU chemotherapy for patients with high grade TS [275, 281-286] while other studies have shown no benefit regarding low grade versus high grade TS [268, 274, 276, 288].

No harmful effect, for the low TS expression group, of adjuvant chemotherapy compared to surgery only was found in our enlarged study in contrast to our previous study [270].

The predictive value of TS is more conclusive in studies (using varying methods, e.g. IHC and TS mRNA with RT-PCR technique) done on metastatic CRC where low TS expression in metastases is associated with a better response to 5-FU [291, 293, 298, 301, 302].

A recent prospective trial, phase II ECOG E4203, randomized first-line treatment for untreated mCRC patients based on tumor TS expression determined by IHC [308]. Patients with low TS expression (grade 0-1) received FOLFOX/Bevacizumab while those with high TS expression (grade 2-3) received irinotecan or oxaliplatin plus bevacizumab or FOLFOX/Bevacizumab. TS was found to be prognostic in that a longer PFS and trend to better OS was found in patients with low grade TS tumors in comparison with patients with high grade TS tumors, regardless of the treatment given in the high grade TS group.

10.2 PAPER II

A Combined Analysis of Mismatch Repair Status and Thymidylate Synthase Expression in Stage II and III Colon Cancer.

Objective: To investigate if a combined analysis of MMR protein expression and TS expression analyzed with IHC in colon cancer has a prognostic value and can predict response to adjuvant 5-FU-based chemotherapy.

Results: In the entire study population of 716 patients with colon cancer, local recurrence occurred in 63 patients (9%) while 187 patients (25%) developed distant metastases. There was a non-significant longer OS for the group of patients receiving postoperative adjuvant chemotherapy (87 months) compared to the group treated with surgery alone (82 months). Of the 327 patients who died in the 120-month follow-up, 270 (83%) died with colon cancer.

Of the 716 patients, 142 (19.8%) were found to have tumors with a dMMR-status with the majority, 125 patients (17.4%), of them having a MLH1-deficiency, 14 (2%) a MSH2-deficiency and 3 (0.4%) were deficient for both MLH1 and MSH2. A dMMR-status was an independent prognostic factor in the entire cohort (HR 0.65, 95% CI 0.34-0.96, p=.007). Patients with dMMR-tumors had an 8-month longer median OS compared to patients with pMMR-tumors. In the group of surgery alone, a dMMR-status remained prognostic (p=0.03) but showed only a trend (p=0.06) in the group receiving postoperative adjuvant

chemotherapy. Although a difference of a 12-month longer median OS was found for patients with pMMR tumors receiving postoperative adjuvant chemotherapy versus those with surgery alone, it was not significant. For patients with dMMR tumors no difference in OS was found comparing the treatment arms.

TS-grade was not prognostic in the entire study population or in the different treatment arms.

In the high TS grade (grade 3), a significant longer OS was observed for the group of patients receiving postoperative chemotherapy compared to group treated with surgery alone in stage II-III (p=0.05) and especially in stage III disease (p=0.004, multivariate analysis).

Four different groups were created based on the combined analysis of MMR-status and TS expression (see Table 8).

Table 8. TS expression (0-2 vs. 3) stratified by MMR status

	dMMR	**pMMR**	**Total**
Low TS Grade 0-2	Group 1: n=84 (12%)	Group 2: n=359 (50%)	n=443 (62%)
High TS Grade 3	Group 3: n=58 (8%)	Group 4: n=215 (30%)	n=273 (38%)
Total	n=142 (20%)	n=574 (80%)	716

Summary of Survival differences in the Groups:

Group 1 (dMMR and Low TS, Grade 0-2): median OS 88 months; no significant difference in OS between the treatment arms (surgery alone arm, n=45; adjuvant arm, n=39) in the entire group or stage-wise.

Group 2 (pMMR and Low TS, Grade 0-2): median OS 84 months was the same for the entire group and subgroups of treatment arms (surgery alone arm, n=186; adjuvant arm, n=173).

Group 3 (dMMR and High TS, Grade 3): median OS 92 months; the 13-month OS difference between the surgery alone arm (n=25, median OS 85 months) and the post-operative adjuvant chemotherapy arm (n=33, median OS 98 months) was not significant.

Group 4 (pMMR and High TS, Grade 3): median OS 75 months; the 21-month OS difference between the surgery alone group (n=114, median OS 67 months) and the post-operative adjuvant chemotherapy arm (n=101, median OS 88 months) was not significant (p=0.09) (Figure 19 A).

Stagewise a significant difference in median OS was found in stage III disease (n=116) for the group receiving post-operative adjuvant chemotherapy, p=0.01 (univariate analysis) (Figure 19 B) as well as in multivariate analyses, p=0.008 (HR, 0.53 [95% CI, 0.33-0.85]) see Table 9.

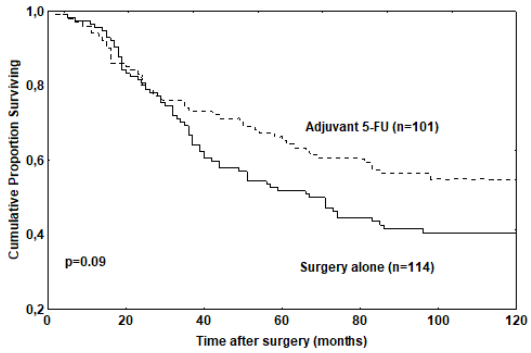


Figure 19 A. Overall survival for patients in Group 4 (MMR proficient tumors with a high TS, grade 3) according to treatment status, (n=215).

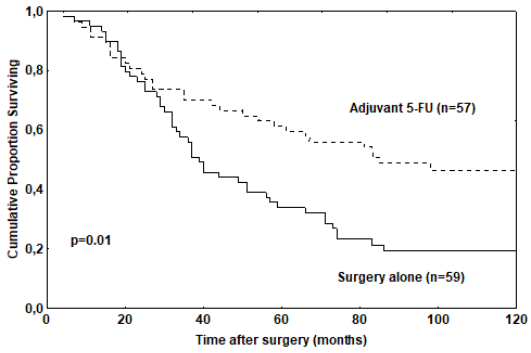


Figure 19 B. Overall survival for stage III patients in Group 4 (MMR proficient tumors with a high TS, grade 3) according to treatment status, (n=116).

Table 9. Group 4 Stage III (n=116) Multivariate analysis using Cox Proportional Hazards Model to identify significant variables and generate HR

Analysis	HR for death	95% CI	p-value
Age (≥66 vs. <66)	1.54	0.96-2.45	NS
Sex (female vs. male)	0.78	0.48-1.28	NS
Grade of differentiation (well/moderate vs. poor)	0.61	0.38-0.97	0.04
Treatment (chemotherapy vs. no chemotherapy)	0.53	0.33-0.85	0.008
Number of analyzed LN (>11 vs. 0-11)	1.52	0.69-3.37	NS
Tumor site (proximal vs. distal)	0.70	0.44-1.12	NS

Abbreviations: MMR, mismatch repair;MMR positive, MLH1 or MSH2 positive, MMR negative, MLH1 negative or MSH2 negative; TS, thymidylate synthase; HR, hazard ratio; 95% CI, 95% confidence interval, proximal;To splenic flexure

Comparison of group 1 and group 4 showed a non-significant difference in OS, p=0.09 in the entire study group. In the subgroup analyses of the surgery alone arm, group 1 had a tendency to an improved OS p=.06 (Figure 20 A) while no significant difference was found in the post-operative adjuvant chemotherapy group (p=0.70) (Figure 20 B).

There was no relationship between MMR-status and TS expression (r=0.03, p=.46)

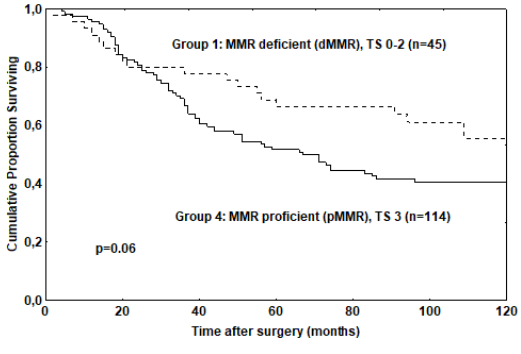


Figure 20 A. Overall survival for patients in Group 1 (MMR deficient tumors with a low TS, grade 0-2) compared to patients in Group 4 (MMR proficient tumors with a high TS, grade 3) treated with surgery alone, (n=159).

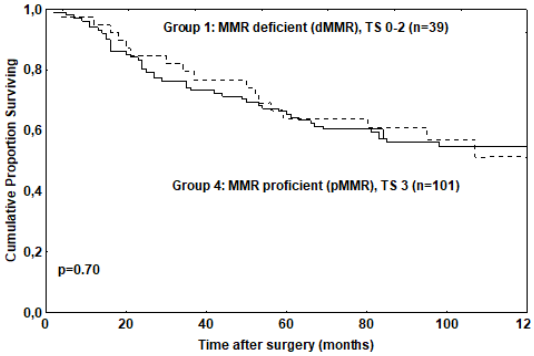


Figure 20 B. Overall survival for patients in Group 1 (MMR deficient tumors with a low TS, grade 0-2) compared to patients in Group 4 (MMR proficient tumors with a high TS, grade 3) treated with surgery and adjuvant chemotherapy, (n=140).

Discussion

A combined analysis of MMR status and TS expression in colon cancer was done to explore if they could together provide information to improve treatment decision in the adjuvant setting.

The prognostic value of MMR status in primary CRC has been established as described by a systematic review with dMMR tumors having an improved prognosis [121]. Concerning the predictive role of MMR-status in stage II-III CRC, most retrospective studies have found that patients with tumors displaying a pMMR-status benefit from adjuvant 5-FU treatment [123, 245, 246].

As mentioned in the discussion of Paper I, low TS expression has been correlated to a better outcome and the majority of retrospective studies indicate a high TS expression as predictive to a better response to adjuvant 5-FU in stage II-IIII CRC.

With the 4 groups classified by TS expression stratified by MMR-status, our study focused on group 1 (patients with dMMR tumors and a low TS, grade 0-2) and group 4 (patients with pMMR tumors and a high TS, grade 3). Our result of no difference found in OS or DFS in group 1 (patients with dMMR tumors and a low TS, grade 0-2) concerning stage or treatment arm seems reasonable based on published data. We found a significant better survival in group 4 (patients with pMMR tumors and a high TS, grade 3), for the patients receiving adjuvant 5-FU treatment in stage III but not in stage II. The better clinical outcome found for group 3 (patients with dMMR tumors with high TS) could be due to an added favorable prognostic factor where group 3 had a higher percentage (20%) of >11 lymph nodes analyzed than the other groups (10%). Conclusion is also hampered by the small size of group 3.

The studies in CRC which analyze TS expression and MMR protein expression as a combined analysis have used varying methods for analyzing TS and MMR, included different stages as well tumor localization (colon vs colorectal) and treatment scenarios (adjuvant and palliative) (see Table 10). Thus, contradictory results arise and comparing the studies is a challenge. A similar study to ours where patients were included in clinical randomized trials with stage II-III colon cancer could not either establish a significant relationship between MMR status and TS expression [437]. No correlation between MMR status and TS expression was also observed in two other studies [438, 439].

Four of the studies found a significant relationship between a high expression of TS and MSI (dMMR) [440-443]. The theory was posed in two of these studies [440, 441] that this relationship could influence the lack of response of dMMR tumors to 5-FU-based-chemotherapy . The larger study of these four studies (n=340) did not find that TS expression was associated with an improved survival or 5-FU resistance for patients with MSI-H/dMMR CRC [443]. All of the four studies included patients with stage IV disease which could influence results as TS expression is a stronger predictive marker in disseminated CRC [291-293]. The differing amounts of high TS, in part due to what accounts as high TS (grade 2-3 versus grade 3), is also a factor to be considered in the interpretation of the results.

The latest study with a combined analysis of TS and MMR is a prospective one using tumor material from two adjuvant trials (Alliance for Clinical Trials in Oncology C9581 and C89803) in colon cancer (in C9581 n=435 stage II; in C89803 n=463 stage III) [279]. The C9581 trial randomized patients to either edrecolomab (antibody inhibiting EpCAM) versus observation alone while the C89803 trial randomized patients to adjuvant 5FU/LV

or 5FU/LV and irinotecan (IFL). Although it was not an independent prognostic factor, they found that high TS expression (grade 2-3) correlated to better outcome (DFS: HR 0.67. 95% CI 0.53-0.84; OS: HR 0.68 95% CI 0.53-0.88) for high versus low TS expression, respectively. Unlike our study, a correlation between high TS expression and dMMR was found p=0.0003. No prediction of benefit of 5-FU-based chemotherapy based on tumor TS expression was found. A limitation in their study is the inability to distinguish stage of disease and treatment.

Table 10. The collected literature of a combined analysis of MMR protein expression and TS expression in colorectal cancer.

Author	Year	[Ref]	No. of pts	Primary tumour	Stage of disease	Method MMR-status	Method TS	% MSI-H /MMRP neg	% TS High	Correlation
Calascibetta et al	2004	[438]	80	CRC	?	PCR	IHC+PCR	MSI:60%	72%	No
Ricciardiello et al	2005	[440]	192	CRC	II-IV	IHC+PCR	IHC	MSI-H:18%, MLH1neg:10%	21%	MSI-H correlates with high TS (p< 0.001)
Popat et al	2006	[439]	441	CRC	I-III	PCR	IHC	MSI:15%	59%	No
Sinicrope et al	2006	[437]	320	Colon	II-III	IHC+PCR	IHC	MSI-H:19%, MMRPneg:19%	75%	No
Odin et al	2007	[441]	181	CRC	I-IV	PCR	PCR	MSI-H: 19%	NE	MSI-H correlates with high TS (p< 0.0001)
Bendardaf et al	2008	[444]	73	CRC	IV	IHC	IHC	NE	NE	MMRP neg correlates with low TS (p=0.0001)
Jensen et al	2008	[442]	130	CRC	I-IV	PCR	IHC	MSI-H:15%	NE	MSI-H correlates with high TS (p< 0.001)
Jensen et al	2009	[443]	340	CRC	II-IV	IHC+PCR	IHC	MSI:14%, MMRPneg:17%	25%	MSI-H correlates with high TS (p=0.001)
Öhrling el al	2013	[445]	716	Colon	II-III	IHC	IHC	dMMR 20%	74%* 38%**	No
Niedzwiecki et al	2017	[279]	898	Colon	II-III	IHC	IHC	dMMR: 18%	52%	dMMR correlates with high TS (p=0.003)

MMR;Mismatch repair, TS;Thymidylate synthase, , No.;Number, pts;patients, CRC;Colorectal cancer, PCR;Polymerase chain reaction, IHC;Immunohistochemistry, MSI-H;Microsateliite instbility-high, MMRP;Mismatch repair protein, NE;Not evaluated, * TS High as grade 2-3; **TS High as grade 3

10.3 PAPER III

Tumor Budding *Versus* Mismatch Repair Status in Colorectal Cancer – An Exploratory Analysis

Objective: To assess tumor budding in primary colorectal tumors with respect to MMR-status and local recurrence/metastases.

Results: For the whole study population of 134 patients we found a 5-year survival rate of 52%, a median OS of 60 months and DFS of 49 months. The median follow-up of surviving patients was 100 months (range 62 to 120 months). OS was not significantly different when comparing stage II and III CRC in the study. Analysis of OS in the four different groups was done with the groups dMMR/met- and pMMR/met- having expected better OS compared to the pMMR/met+ and dMMR/met+ groups (Figure 21).

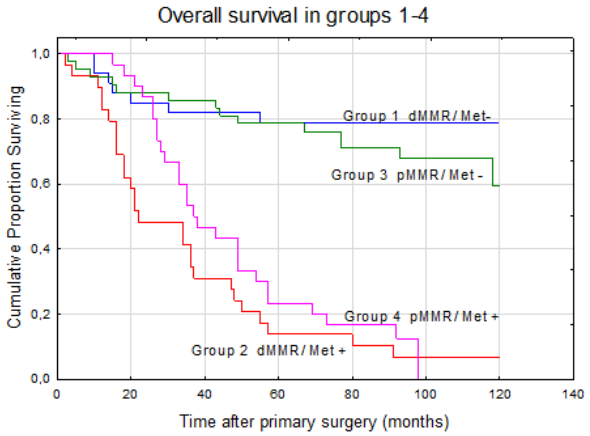


Figure 21. Overall survival in the four different groups.

The distribution of dMMR tumors was higher proximal to the splenic flexure 40/71 (56%) compared to tumors distal to it 22/63 (35%). Analyses of the Met+ population (n=59) showed that the dMMR group had a higher number of patients (15/29) (52%) who had a local recurrence or metastases in the first year compared to the pMMR group (4/30) (13%) but with no significant difference in time to recurrence (p=0.4).

In this study the tumor budding mean was 7 (SD±6), the median 5 and the range 0-35. Agreement of tumor budding assessment was substantial between the investigators (p<0.001, r=1.0, ICC=0.99).

Using the cut-off of 5 and 10, a significantly higher frequency of high-grade budding was observed in the dMMR/met+ group (cut-off 5, 72%; cut-off 10, 45%) versus the dMMR/met- group (cut-off 5: 39%, cut-off 10: 21%) (p=0.009 and p=0.047, respectively). This was also observed in the dMMR/met+ (cut-off 5: 72%, cut-off 10: 45%) versus the pMMR/met+ group (cut-off 5: 40%; cut-off 10: 17%) (p=0.01 and p=0.02 respectively) as well as compared to the pMMR/met- group using the cut-off 10: 19%, p=0.012. For more information on the distribution of tumor budding between the four groups see Table 11.

Table 11. Distribution of tumor budding in Groups 1-4

Method	**Total n=134**	**Group 1 (dMMR/met-) n=33**	**Group 2 (dMMR/met+) n=29**	**Group 3(pMMR/met-) n=42**	**Group 4 (pMMR/met+) n=30**
TB cut-off 5:					
Low (<5)	64(48%)	20(61%)	8(28%)	18(43%)	18(60%)
High (≥5)	70(52%)	13(39%)	21(72%)	24(57%)	12(40%)
TB cut-off 10:					
Low (<10)	101(75%)	26(79%)	16(55%)	34(81%)	25(83%)
High (≥10)	33(25%)	7(21%)	13(45%)	8(19%)	5(17%)

Abbreviations: TB, tumor budding; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair; met-, no recurrence or distant metastases; met+, with recurrence or distant metastases

In the pMMR category as a whole, we found 50% and 18% had high-grade tumor budding using the cut-off of 5 and 10 respectively, compared to the dMMR category which had 55% and 32%, respectively.

Regardless of cut-off used we found no significant survival advantages with regard to tumor budding grade.

Discussion

This exploratory study found a significantly higher frequency of tumor budding in the dMMR/met+ group compared to the other 3 groups including pMMR/met+. Using the 2-cut-offs for high-grade versus low-grade we found no significant survival advantage for the low-grade tumor budding group. This is not surprising since the study is a small study designed to evaluate differences in tumor budding in prearranged subgroups. Our number of 35 patients with dMMR tumors who developed recurrence/metastases out of 1006 patients reflects the inherent biology of dMMR primary CRC with a known favorable prognosis.

High-grade tumor budding is believed to reflect cell de-differentiation which in turn has been correlated with the development of regional and distant metastases and a worse prognosis [310, 333, 446]. The process of cell de-differentiation can be observed in the epithelial-mesenchymal transition (EMT) and the activation of the Wnt signaling pathway [310, 447, 448]. CRC with a pMMR status is regarded as chromosomally unstable, commonly harbors an APC mutation and is driven by activation of the Wnt pathway [447]. This mechanism is thought to be the explanation behind the general finding of pMMR CRC having more high-grade tumor budding compared to dMMR CRC [333]. Tumor budding and its negative prognostic impact has been found in some studies to be independent of MMR-status [348, 449].

Our finding that dMMR/met+ had more high-grade tumor budding than pMMR/met+ could be due to the study design which may include a higher number of dMMR cases with recurrence or metastases than other studies. Table 12 summarizes studies which analyzed MMR-status with regard to tumor budding. Two studies used H&E stained slides [357, 449] while our study and Lugli et al's [348] used cytokeratin 22 staining. Different assessment methods and cut-offs were used in the studies.

Table 12. Clinicopathological studies analyzing high grade vs low grade tumor budding with regards to MMR-status

Author & cut-off		**pMMR**	**dMMR**
Lugli[348]	≤6 low PTB	21%	34%
	>6 high PTB	79%	66%
	≤6 low ITB	56%	64%
	>6high ITB	44%	36%
Kevans[357]	*low PTB	52%	74%
	**high PTB	48%	26%
Karlberg[450]	<5 low PTB	50%	45%
	≥5 high PTB	50%	55%
	<10 low PTB	82%	68%
	≥10 high PTB	18%	32%
Wyk[449]	<20 low PTB	15%	85%
	≥20 high PTB	85%	15%

Abbreviations: pMMR, proficient mismatch repair; dMMR, deficient mismatch repair; PTB, peritumoral budding; ITB, intratumoral budding; SD, standard deviation; ND, no data given; *low PTB (median tb=0); ** high PTB (median ≥1)

There may be other mechanisms contributing to tumor budding in dMMR CRC than the Wnt signaling. One study found that dMMR tumor buds had lower β-catenin expression compared to buds found in pMMR tumors and that in especially CRC with MLH1-loss, high-grade tumor budding may have increased vessel involvement and nodal metastases rather than local invasiveness [451].

The cut-offs of 5 and 10, defining high-grade versus low-grade budding, used in our study were also used in other studies, with cytokeratin staining [340] and with H&E stained sections [309, 367]. Comparison of our results with other studies is difficult due to the wide heterogeneity in assessment methods and cut-off values.

10.4 PAPER IV

Prognostic value of Tumor Budding, Tumor Border Configuration and T-cell infiltration in Colon Cancer

Objective: To examine the prognostic impact of tumor budding, tumor border configuration as well as T-lymphocyte density (CD3+ and CD8+) in primary colon cancer with known MMR-status.

Results: The patients median age was found to be 66-years-old and a similar distribution was found for gender, colon site, stage II and III and treatment arm when patient and tumor characteristics were stratified by tumor budding grade status and T-lymphocyte (CD3+ and CD8+) score. MMR-status was not available for 24 patients (5%). Of the tumors with known MMR-status, 364 (80%) were pMMR and 90 (20%) were dMMR. In the dMMR category most 57/90 (63%) were located in the right colon, 15/90 (17%) in the transverse colon and 18/90 (20%) in the left colon compared to the pMMR category which had 151/364 (41%), 31/364 (9%) and 182/364 (50%), respectively.

In the whole patient material, 192 (40%) of the tumors had an infiltrating tumor border configuration while 286 (60%) had a pushing (181, 38%) or mixed (105, 22%) border configuration. A higher percentage of infiltrating border configuration was found in pMMR (160/364, 44%) tumors compared to dMMR tumors (22/90, 24%) ($p<0.001$).

Tumor budding analysis of the 478 patients showed a mean budding count of 7.6 (SD±6), a median count of 5.85 and a range of 0 to ≥35. The agreement on tumor budding count between the investigators was substantial ($p=0.006$, r=0.99; ICC=0.99). With high grade tumor budding defined as ≥10 buds, we found 132/478 (28%) in this category and stagewise, 92/253 (36%) in stage III and less 40/225 (18%) in stage II ($p<0.001$). No significant distribution difference between high grade tumor budding or low grade was found when looking at tumor site and T-stage. There were significantly more tumors in the pN1-2 group with high grade tumor budding compared to the pN0 group, 38% and 18% respectively ($p<0.001$). A tendency to more high-grade tumor budding was seen for the G3 tumors (poorly differentiated) (36%) versus the G1 (18%) and G2 (27%) tumors ($p=0.068$). Non-mucinous tumors had 31% high grade tumor budding compared to the mucinous tumors with 13% ($p<0.001$).

The infiltrating tumor border configuration tumors had 98/192 (51%) with high grade tumor budding while the pushing/mixed tumor border configuration had 34/286 (12%) ($p<0.001$). This was consistent stagewise (stage III: 67/120, 56% versus 25/133, 19%, respectively, $p<0.001$; stage II: 31/72, 43% versus 9/153, 6%, respectively, $p<0.001$).

Analysis of MMR-status and tumor budding showed that pMMR tumors had more high grade tumor budding 109/364 (30%) than dMMR tumors 17/90 (19%) (p=0.035).

When exploring tumor budding and T cell (CD3+, CD8+) scores we found that high grade tumor budding was associated with low total CD3+ and CD8+ scores (CD3+: p=0.0398; CD8+: p=0.026). See Table 13.

Table 13. Tumor budding and total CD3+ and CD8+ score.

		Tumor Budding Frequency N (%)		
	Frequency N (%)	**Low (< 10 buds) n=346**	**High (≥ 10 buds) n=132**	***P***
CD3 total:				0.039
low	113 (24%)	84 (24%)	29 (22%)	
intermediate	237 (49%)	160 (46%)	77 (58%)	
high	128 (27%)	102 (30%)	26 (20%)	
CD8 total:				0.026
low	148 (31%)	105 (30%)	43 (33%)	
intermediate	222 (46%)	152 (44%)	70 (53%)	
high	108 (23%)	89 (26%)	19 (14%)	

There were more high CD3+ and CD8+ score tumors in proximal colon tumors (CD3+: 30%; CD8+: 25%) compared to distal ones (CD3+: 23%; CD8+: 19%) (CD3+: p=0.003; CD8+: p=0.220). A higher percentage of high CD3+ score (41%) and CD8+ score (40%) was observed in poorly differentiated tumors (G3) compared to well differentiated (G1) (CD3+: 20%; CD8+: 13%) and intermediate (G2) ones (CD3+: 23%; CD8+: 19%), (CD3: p=0.004; CD8: p<0.001). In non-mucinous colon tumors, a high score CD3+ of 30% and CD8+ of 25% was observed compared to mucinous tumors that had 14% and 8%, respectively (CD3: p<0.0001; CD8: p<0.001).

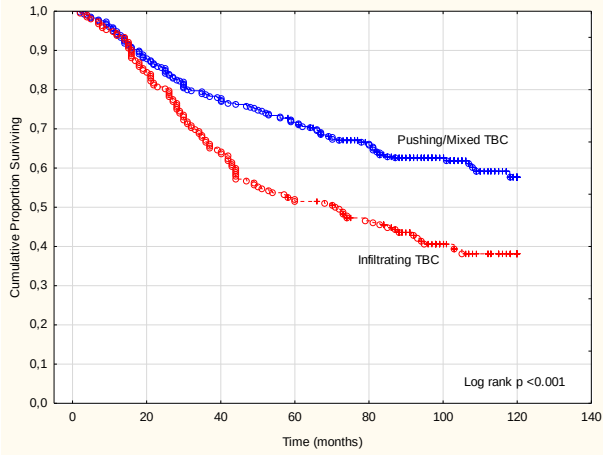
A significantly higher amount of high score CD3+ (p<0.001) and CD8+ (p<0.001) was found in dMMR tumors (CD3: 39/90, 43% and CD8: 33/90 37%) compared to pMMR tumors (CD3: 77/364, 21% and CD8: 66/364, 18%). The pushing/mixed tumor border configuration tumors had more high score CD3+ (34%) and CD8+ (29%) than the infiltrating tumors (CD3+: 17% and CD8+: 13%) (CD3+: p<0.001; CD8+: p<0.001).

Of the 478 patients, 42 (9%) were diagnosed with local tumor recurrence and 128 (27%) with distant metastases. A 5-year survival rate of 64% was found and the median DFS and OS was 78.5 months and 83 months, respectively. Of the 223 (47%) patients that died in the entire study group during the 120-month follow-up, 181 (81%) were found to have died with colon cancer.

As data from our previously reported studies including MMR status [236] and the original Nordic trials [426] did not find any major difference between DFS and OS this study concentrates on data related to OS.

A significant OS benefit was found for patients that were younger (<66-years-old) (p=0.040), had stage II versus stage III (p<0.001), had dMMR tumors (p=0.038) and had a N0 status (p<0.001). In this study no OS benefit was found for tumor location, treatment arm, tumor grade, mucinous status, gender and only a trend of benefit was found for T0-2 stage versus T3-4 (p=0.0516).

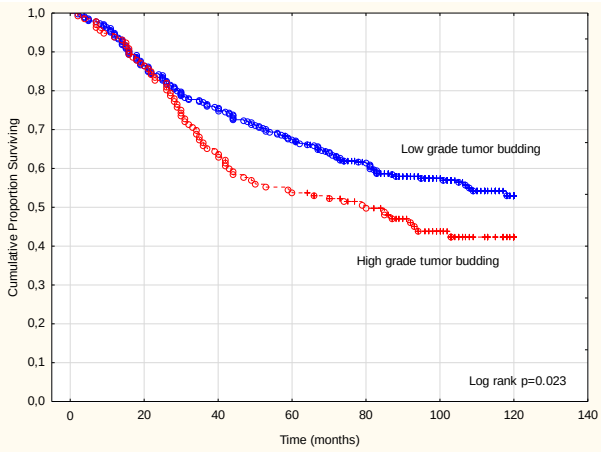
In OS analysis regarding tumor border configuration, tumor budding and CD3+ / CD8+ score, we found a prolonged OS for patients with pushing/mixed border configuration tumors (p<0.001), for ones with low grade tumor budding tumors (p=0.016) and for patients with high total CD8+-score tumors (p=0.013) (Figure 22 A, B, D). For the patients with a high CD3+ score a tendency to a better OS was found (p=0.076) (Fig 22 C). A significantly better OS remained for low grade tumor budding in stage II patients (p=.079) but not for stage III. For patients with right-sided tumors, but not with left-sided tumors, a high CD3+ and CD8+ score was associated with a better OS in univariate analysis (CD3+: p=0.002; CD8+: p<0.001) but not in multivariate analysis (CD3+: p=0.792; CD8+: p=0.066).



Numbers at risk

Pushing/mixed	286	252	223	204	167	103	32
Infiltrating	192	163	124	100	83	39	13

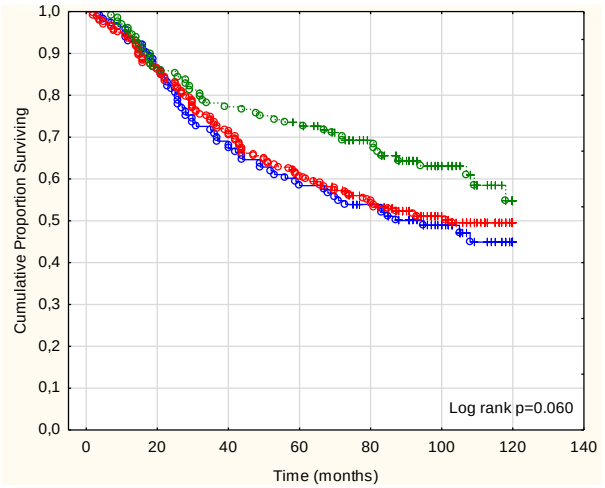
22A) Overall survival according to tumor border configuration (TBC)



Numbers at risk

Low grade tb	346	300	262	232	190	108	38
High grade tb	132	115	85	72	60	34	7

22B) Overall survival according to tumor budding grade

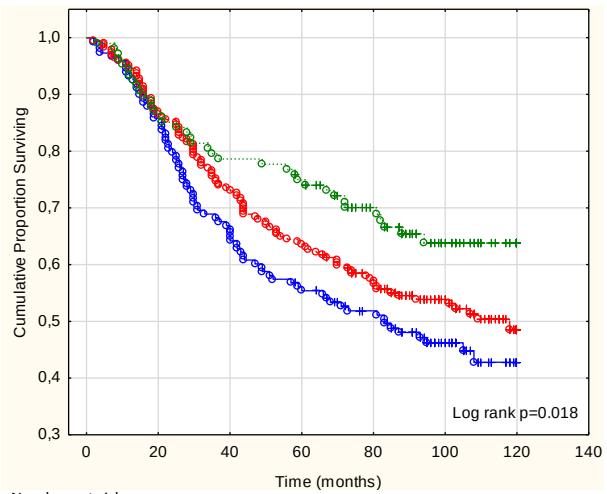


Numbers at risk

Low CD3 score	113	98	78	67	57	36	9
Intermediate CD3 score	237	206	170	144	121	67	22
High CD3 score	128	111	99	93	72	39	14

22C) Overall survival according to CD3-total score
Low total CD3 score = 3-4
Intermediate total CD3 score = 5-6
High total CD3 score = 7-12

Low CD3 score
Intermediate CD3 score
High CD3 score



Numbers at risk

Low CD8 score	148	127	99	82	69	41	11
Interm CD8 score	222	194	163	142	119	70	23
High CD8 score	108	94	85	80	62	31	11

22D) Overall survival according to CD8-total score
Low total CD8 score = 3-4
Intermediate total CD8 score = 5-6
High total CD8 score = 7-12

Low CD8 score
Intermediate CD8 score
High CD8 score

Figure 22 A-D

Adjusting for age, sex, tumor grade, MMR status and T-stage, multivariate analysis showed that OS remained significant for patients with pushing/mixed tumor border configuration tumors and for ones with high CD8+ scores (see Table 14). This was the case even for patients with stage II tumors (TBC: HR=1.96, 95% CI: 1.24-3.09, p=0.004; CD8+: HR0.61, 95% CI:0.44-0.86, p=0.005) but not for ones with stage III tumors. Tumor budding and CD3+ scores did not remain significant in multivariate analysis.

Table 14. Univariate and Multivariate Analyses for Overall Survival using Cox Proportional Hazards Model

Characteristic		**Univariate Analysis OS**		**Multivariate Analysis OS**	
	Events/ patients	**HR (95% CI)**	**p-value**	**HR (95% CI)[a]**	**p-value**
TBC:					
pushing/mixed vs.	110/286	1		1	
infiltrative	113/192	1.79 (1.37-2.32)	<0.001	1.62 (1.21-2.16)	0.001
Tumor budding:					
Low vs.	150/346	1		1	
High	73/132	1.41 (1.07-1.87)	0.016	1.20 (0.88-1.62)	0.247
CD3 score:					
Low (3-4)	59/113	1		1	
Intermediate (5-6)	116/237	0.93 (0.68-1.27)		1.03 (0.73-1.45)	
High (7-12)	48/128	0.66 (0.45-0.97)	0.076	0.69 (0.45-1.05)	0.110
CD8 score:					
Low (3-4)	80/148	1		1	
Intermediate (5-6)	106/222	0.81 (0.61-1.08)		0.87 (0.64-1.20)	
High (7-12)	37/108	0.56 (0.38-0.82)	0.013	0.55 (0.36-0.85)	0.026

[a]p values from multivariate analysis adjusted for age, sex, tumor grade (G1, G2, G3), Stage (II, III), MMR-status (proficient vs. deficient) and T-stage (0-2 vs 3-4). Cases with unknown information on differentiation grade and MMR-status were not included in the multivariate model.
Abbreviations: OS, overall survival; TBC, tumor border configuration; MMR, mismatch.

In the dMMR category, OS analysis with regard to tumor budding, tumor border configuration and CD8+ score was not prognostic. In univariate analysis a high CD3+ score was associated with a better OS (p=0.027) but not in multivariate analysis.

In the pMMR category, patients with low-grade tumor budding had a better OS in univariate (p=0.021) but not in multivariate analysis while patients with a pushing/mixed tumor border configuration had an improved OS in univariate (p<0.001) as well as multivariate analysis (p<0.0001). Analysis of CD3+ and CD8+ was not prognostic in this category and showed only a tendency to better OS in univariate analysis for patients with high CD8+ score tumors (p=0.051).

The median OS between low versus high CD8+ scores for the patients with dMMR tumors was 95 months versus 92 months and for patients with pMMR tumors it was 68,5 months versus 83,5 months.

No significant difference in OS was found between the treatment arms when analyzing tumor budding grade, tumor border configuration as well as CD3+ and CD8+ scores. Only

a tendency to improved OS was noted for patients in the adjuvant chemotherapy arm and high CD8+ score tumors (p=0.075).

Discussion

The focus of this study was to analyze in stage II-III primary colon cancer whether tumor budding, tumor border configuration and T-lymphocyte infiltration with CD3+ and CD8+ cells are prognostic factors.

A main finding in the entire study group of 478 patients and in the stage II group (n=225) of patients, was that tumor border configuration as well as the T-lymphocyte CD8+ score were independent prognostic factors. High grade tumor budding compared to low grade tumor budding was found to be associated with a more advanced stage, a higher N-stage, a pMMR-status, an infiltrating tumor border configuration and a lower level of CD3+ and CD8+ T-lymphocyte infiltration. Tumor budding was found to be prognostic in univariate analysis but not in multivariate analysis.

As in our study, other studies in stage II-III colon cancer have also found an infiltrative tumor border configuration to be prognostic of worse outcome compared to a pushing or mixed tumor border configuration [369, 375]. Our study also further confirmed other studies findings of a correlation between an infiltrative tumor border configuration and high degree tumor budding in colon cancer [309, 358, 447].

The use of cytokeratin-staining to better define tumor buds and the cut-off of 10 to define low versus high-grade tumor budding was also used in other recent studies [358, 452, 453]. One of these studies by Mehta et al 2018 in colon cancer, stage I-IV and using 1-HPF method, found as in our study a similar amount of high grade tumor budding in stage II-III (30% compared to our 28%) and no significant correlation of tumor budding to OS [452]. The two other studies applied the 10-HPF method by Karamitopoulou [340] as we did with the cut-off of 10 [358, 453]. In the Koelzer et al study [358], the amount of high grade tumor budding in the combined stage II-III CRC prospective cohort (n=215) was similar to our study's (30.9% and 28%, respectively). However, in their retrospective stage II CRC cohort (n=150), they had a higher amount of high-grade tumor budding (30.7%) than in our study (18%). This could have contributed to their finding of a significant correlation between high grade tumor budding and DFS. The Eriksen et al study [453] on stage II colon cancer (n=573), found a mean tumor budding of 4.5±3.7 (range 0-25) which is lower compared to ours of 7.6 and other studies [358]. This could have attributed to the study's low amount of high-grade tumor budding (7%) as well as their finding tumor budding to be a non-significant prognostic factor. They found as in our study a significant correlation of high-grade tumor budding and pMMR-status.

The better prognosis associated with a dMMR-status or MSI-H status in primary CRC has been established by several randomized trials and a meta-analysis [121]. This positive effect has been attributed to the association of a dMMR-status with a higher level of T-cell infiltration compared to pMMR tumors [72, 243]. The mechanism behind it is believed to be the higher mutational load found in dMMR tumors resulting in a neo-antigen increase thus triggering the immune response.

However, studies have also shown that regardless of MMR-status, the peritumoral immune infiltrate including T-cell infiltration is prognostic in CRC [418, 454, 455].

Assessment of inflammatory infiltration has been done with different methods in studies. The Klintrup-Mäkinen method grades the inflammatory reaction in a semi-quantitative manner in H&E slides using a 4-point scale ranging from low grade to high grade [415, 416]. The validated immunoscore method utilizes as in our study method, staining of CD3+ and CD8+ T-cells and semi-quantitatively grades regions within the invasive margin and tumor center by using, unlike our study, digital pathology. In the IS method scoring groups range from 0 to 4, from lowest density to highest density, where the calculated score for each case is placed in the appropriate scoring group [456].

This study's T-cell CD3+ and CD8+ infiltration was assessed according to the method used by Dahlin and Ogino [417, 418].

A study by Berntsson et al used the same method as ours to analyze T-cell CD3+ and CD8+ infiltration in stage I-IV CRC but in TMA rather than whole tissue [457]. Stratifying by tumor location, they found high CD8+ density was an independent favorable prognostic factor for patients with right-sided colon tumors and high CD3+ cell density for the right colon and the rectum. Unlike our study they found a significant prognostic interaction between CD8+ and right-sidedness (p=0.031).

One study by Lugli et al, used as in our study immunostaining to analyze tumor budding and the peritumoral immune infiltration of CD8+ T-cells (ref 26) in CRC (stage II-III, n=273) [458]. Differing from our study, counting of tumor buds as well as CD8+ cells was done in one-HPF with a cut-off, derived by ROC curve analysis, of 16 for tumor budding grade and of 40 for high density CD8+. They found a higher amount of high-grade tumor budding (stage II-III 33%; stage II 24%; stage III 42%) than our study did (stage II-III 28%; stage II 18%; stage III 36%). Similar to our study, tumor budding was prognostic in univariate analysis but not in multivariate analysis. However, they found that a CD8+/tumor buds index was an independent prognostic factor in which a low index was associated with worse survival.

Another study that also studied tumor budding using the method of Lugli et al [458], MMR-status and the immune infiltration of CD8+ T-cells as well as FOXP3+ (T regs) and CD68+ T-cells was done by Zlobec et al 2011 in stage II-III CRC (n=297) [459]. Controlling for prognostic factors that included MMR-status, multivariate analyses showed an independent correlation of higher numbers of tumor buds with worse outcome and of higher numbers of CD8+, FOXP3+ and CD68+ cells to an improved outcome.

10.5 Limitations (PAPER I-IV)

With the use of IHC there are pitfalls such as poor internal control, heterogeneous staining patterns, overstaining which limits evaluation or results in false positive interpretation and weak staining leading to false-negative interpretation. Necrotic tumor areas are problematic because they can show false-negative as well as non-specific positive staining. There is always a risk of misclassification when dealing with semi-quantitative methods. Our application of a standardized defined manner and area of measuring and independent observers blinded to clinical data was done to help minimize this.

Our tumor material derived from the 2224 patients, included 1991-1997 with primary CRC included in the adjuvant Nordic trials randomized to surgery alone versus surgery followed by 5-FU-based adjuvant chemotherapy [426]. It is important to note that this Nordic trial

found no significant survival advantage in the entire study population. A non-significant difference of 7% was found in favor of adjuvant chemotherapy for colon cancer stage III. This has to be taken into account in analysis of our data, as well as the limitation of few lymph nodes (<12) analyzed in the Nordic trials for the majority of the cases with thus a risk of understaging.

Retrospective studies can have an unseen selection bias.

Tumor material could during prolonged time have a potential loss of antigenicity due to oxidation of cut sections.

Interpreting the results of studies examining the prognostic or the predictive value of TS expression in CRC is complicated since there may be publication bias, underpowered studies, heterogeneity with methodology including use of different types of antibodies in IHC and TS scoring between the studies. Even factors, such as surgically induced ischaemia during resection of tumors can possibly have altered TS expression and impact the result of TS testing [460]. In our study, a limitation in the TS expression analyses could be the time span between the previously analyzed tumor material from 862 patients and the analyses done later on with 527 additional patients.

Tumor budding has not always been found to be an independent prognostic factor in CRC studies, most likely due to the studies having varied cohorts, different methods and cut-offs to define high-grade budding. In our two tumor budding studies, the 10-HPF method adapted from Karamitopoulou et al [340] used in Paper IV combined with the use of digital pathology was a more reliable method than the one used in Paper III.

11 CONCLUSION

There is a remaining need for a better risk stratification for Stage II and even Stage III CRC patients to improve adjuvant treatment selection. Our studies were done to explore whether TS expression, MMR expression, tumor budding, tumor border configuration and T-cell infiltration (CD3+, CD8+) could contribute to a better risk stratification for these patients. The following conclusions could be made from the studies:

Paper I:
We demonstrated that TS expression, assessed by IHC, is an independent prognostic factor in the group of patients with primary CRC treated with surgery alone as well as showing a benefit of adjuvant 5-FU-based chemotherapy in patients with high TS expression.

Paper II:
Considering the heterogeneity of the disease with different subsets within stage III (IIIA, IIIB, IIIC) the question arises whether all patients in stage III benefit from 5-FU-based adjuvant chemotherapy. In our study we found that a combined analysis of MMR status and TS expression can aid in the prediction of response to 5-FU-based-chemotherapy in stage III colon cancer.

Paper III:
In our study we found a significantly higher grade of tumor budding in dMMR/met+ CRC. Tumor budding can be of prognostic importance in dMMR CRC.

Paper IV:
In our study on primary colon cancer, stage II-III, we found an independent prognostic impact of CD8+ lymphocyte infiltration and tumor border configuration and an association between them and tumor budding. Our study supports the inclusion of tumor border configuration, tumor budding, CD8+ T cell infiltration in the risk assessment for stage II-III colon cancer patients.

12 FUTURE PERSPECTIVES

Further research into prognostic and predictive factors is required to help identify patients with stage II-III CRC at risk for disease recurrence and to select the most suitable treatment for the individual patient.

To unequivocally establish TS expression in CRC as a prognostic factor, prospective studies are needed with homogenous CRC groups analyzed as well as standardized unbiased methods and assessment in which investigators are blinded to clinical data. Studies on the predictive effect of TS expression on adjuvant 5-FU-based chemotherapy should also take into account other enzymes involved in 5-FU metabolism (e.g. DPD, TP).

A combined marker analysis of MMR status and TS expression can contribute to tumor-node-metastasis staging, especially for stage II-III, when making treatment decision for these patients.

Further, primary CRC studies should be done using tumors from a larger patient group, to assess tumor budding with an established method and cut-off so as further establish whether tumor budding is a negative prognostic factor in CRC, including stage II dMMR CRC.

Varied study cohorts, different tumor budding methods and cut-offs for high grade budding has contributed to the inconsistency of tumor budding not always remaining an independent prognostic factor in CRC studies. The recently proposed tumor budding assessment advocated by ITBCC using H&E slides and the 3-tier scheme can be the established method used in future studies and should also ease incorporating assessment of tumor budding as a routine in clinical practice. A future goal is to establish tumor budding as a prognostic tool for stage I-II-III CRC.

Tumor budding has also been correlated to KRAS and BRAF mutations and independently predicted poor outcome in all CRC stages [461].

There is an increased awareness of the impact tumor microenvironment in CRC, including tumor stroma and tumor-infiltrating lymphocytes (TILS) has on prognosis and the response to oncological treatment. Our study, Paper IV, contributes to the evidence in primary colon cancer, that the adaptive T-lymphocyte infiltration, especially the cytotoxic CD8+ T-lymphocytes, is an independent prognostic factor. Hopefully, prospective studies can further determine whether patients with high-risk stage II and stage III colon cancers with a high CD8+ score benefit more from adjuvant chemotherapy than patients with a low CD8+ score.

Clinicians have used TNM-staging including stage, risk factors such as vessel involvement, perineural involvement, grade of differentiation and MMR-status to determine which patients could benefit from adjuvant chemotherapy. There are only three biomarkers, MMR-status, RAS-status and BRAF-status that are available for clinical routine use to aid in determining the prognosis of a patient and predicting treatment therapy.

There is a high level of heterogeneity, both genomic and transcriptomic, in CRC which poses a challenge in finding additional reliable biomarkers. The Consensus Molecular Subtypes (CMS) was developed for CRC in an effort to aid in prognostication, to identify the patients who would benefit of adjuvant chemotherapy as well as to individualize the oncological treatment in all stages [462]. Based on gene expression data, 4 different groups are distinguished: CMS1 (MSI immune), CMS2 (Canonical), CMS3 (Metabolic), CMS4 (Mesenchymal). Tumors in the CMS1 subtype are more often observed in the female gender, have a high histological grade and are associated with dMMR-status or MSI-H,

BRAF mutation, right-sided colon location and an anti-tumor immune environment. The CMS2 subtype is the most heterogeneous one of the four subtypes and the CMS3 subtype is associated with KRAS mutations. More advanced stages are observed in the CMS4 subtype which is characterized as pro-inflammatory and by mesenchymal and stromal gene signatures rather than signatures from the cancer cell itself. An increased number of tumor buds was observed in CRC classified in the CMS4 subtype versus tumors classified in CMS2 and CMS3 subtype [461]. See Table 15 for summary of CMS subtypes.

Table 15. Consensus molecular subtypes of colorectal cancer. (Reprinted, with permission, from Guinney et al, Nat Med 2015).

CMS1 MSI Immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGFβ activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

CIMP, CpG island methylator phenotype; MSI, microsatellite instability; SCNA, somatic copy number alterations.

Analyses of the original dataset used to create CMS shows that distributions differ in early compared to metastatic disease at diagnosis (early stage: CMS1 16%, CMS2 43%, CMS3 15%, CMS4 26% versus stage IV: CMS1 8%, CMS2 43%, CMS3 9%, CMS4 40%) [463]. There were about 13% of the tumors in the original dataset that did not classify into a specific subtype probably due to intra-tumoral heterogeneity.

The CMS subtype classification in CRC can be useful in the design of new clinical trials to identify reliable biomarkers where patients should be stratified in the correct clinical context (early stage versus stage IV).

The future of biomarkers in the CRC adjuvant setting, especially relevant for stage II, may be a combination of markers (TS, MMR, tumor budding, CD8+ T cell infiltration) that contribute to a prognostic/predictive score which will aid the oncologist in designing the patient's individual treatment plan.

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